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Epidemiological and molecular insights into Human Papillomavirus-related head and neck squamous cell carcinomas

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Epidemiological and molecular insights into Human Papillomavirus-related head and neck squamous cell carcinomas

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List of Abbreviations

AJCC	American Joint Committee on Cancer
AP	Activator protein
aPKC	Atypical protein kinase C
BSA	Bovine serum albumin
CAL	Cystic fibrosis transmembrane conductance regulator-associated ligand
CBP	CREB binding protein
CBP/p300	CREB binding protein
CDK	cyclin dependent kinase
CDK1	cyclin dependent kinase inhibitor
CFTR	Cystic Fibrosis Transmembrane Regulator
CI	Confidence Interval
CIN	cervical intraepithelial neoplasia
CR	conserved regions
DMEM	Dulbecco's Modified Essential Medium
DSS	disease specific survival
E6AP	E6 associated protein
EB	ERM binding region
EBP50	Ezrin-Radixin-Moesin Binding Protein 50
ECOG	Eastern Cooperative Oncology Group
ECOG 1308	Eastern Cooperative Oncology Group Trial
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal-Transition
ER	Estrogen Receptor
ERE	Estrogen Response Elements
ERM	Ezrin-Radixin-Moesin
FADD	Fas associated death domain
FFPE	Formalin Fixed Paraffin Embedded
GRK6A	G protein coupled receptor kinase A
GRO	General Register Office
GST	Glutathione S Transferase
HDAC	histone deacetylases
hDlg	human homologue of Drosophila disc large protein
HIFBS	heat inactivated Foetal Bovine Serum
HNSCC	head and neck squamous cell carcinomas
HPV	Human Papillomavirus
HR	Hazard Ratio
hScrib	human homologue of the Drosophila scribble protein
hTERT	human telomerase reverse transcriptase
IGFBP3	Insulin growth factor binding protein-3
IHC	Immunohistochemistry
INHANCE	The Head and Neck Epidemiology Consortium
IRF-1	interferon regulatory factor-1
ISH	In Situ Hybridisation
JAK/STAT	Janus kinase/signal transducers and activators of transcription
MAGI	Membrane Associated Guanylate Kinase Inverted
MAPK	mitogen-activated protein kinase
MATH	mutant-allele tumour heterogeneity
MEF	mouse embryonic fibroblasts

MHC-1	Major Histocompatibility Complex
MUPP-1	Multiple PDZ domain protein-1
NICE	National Institute of Clinical Excellence
OKCs	oral keratinocytes
OPSCC	oropharyngeal squamous cell carcinomas
ORF	open reading frame
OS	overall survival
PAF	Population Attributable Fraction
Pals1	protein associated with Lin seven 1
PatJ	PALS1-associated tight junction protein
PBM	PDZ binding domain
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDGF	platelet derived growth factor
PDGFR	Platelet Derived Growth Factor Receptor
PDZ	PSD-95 - a 95 kDa protein involved in signalling at the post-synaptic density, DLG - the Drosophila melanogaster Discs Large protein and ZO-1 the zonula occludens 1 protein
PI3K	Phosphoinositide 3 kinase
PI3K/Akt/mTOR	Phosphoinositide 3-kinase/ serine-threonine protein kinase B/mammalian Target Of Rapamycin
PKA	Protein Kinase A
pRB	Retinoblastoma protein
PsVs	pseudovirions
PTEN	Phosphatase and tensin homologue
qRT-PCR	quantitative Reverse Transcriptase PCR
RFS	recurrence free survival
RT-PCR	Real time Polymerase Chain Reaction
RTOG	Radiation Therapy Oncology Group
Scrib	Scribbled planar cell polarity protein
TAE Buffer	Tris-acetic acid-EDTA
TCF/LEF	T-cell factor/lymphoid enhancer factor
TE	Tris-EDTA
TGFα	Transforming Growth Factor α
TIP-2/GIPC	Tax Interacting Protein, clone 2/GAIP Interacting Protein, C terminus
TLR9	toll-like receptor 9
TORS	Trans Oral Robotic Surgery
TTB	Tayside Tissue and Tumour Bank
UICC	Union for International Cancer Control
WHO	World Health Organization

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Declaration

I declare that I am the sole author of this thesis and that all of the references cited in this thesis have been consulted by me, unless otherwise indicated. The thesis is written in my own words and conforms to the University of Dundee's Policy on Plagiarism and Academic Dishonesty. The work presented in this thesis has been done by me, unless otherwise acknowledged and this work has not previously been submitted for consideration for any other higher degree.

Signed……..

Athiva Shankar

Date…….

Certificate

I hereby certify that Athiva Shankar has fulfilled the condition of Ordinance 39 of the University of Dundee and is qualified to submit this thesis for the degree of doctor of philosophy.

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Summary

Over the last decade, Scotland has witnessed a rising incidence in squamous cell carcinomas of the head and neck (HNSCC), a phenomenon thought to be linked to infection with high-risk Human Papillomavirus (HPV). HPV-associated HNSCC are a distinct disease presenting unique epidemiological, biological and clinical challenges. However, establishing HPV-related disease is impaired by non-standardised testing protocols and lack of a consensus on the efficacy of existing biomarkers such as p16. This is further complicated by the absence of additional biological markers and a dearth in our understanding of the molecular mechanisms underlying HPV-driven tumourigenesis. While HPV positivity is more commonly detected in the oropharynx, its prevalence and clinical impact in other head and neck subsites remains largely unexplored. The research presented in this thesis was undertaken to determine the prevalence of high-risk HPV in a heterogeneous cohort of 293 HNSCC patients from Tayside and to evaluate the validity of EBP50, a scaffolding protein involved in cell polarity which is targeted by high-risk HPV, as a potential marker for HPV-driven HNSCC.

The p16 status of the patients in the cohort was already known and tissue specimens were genotyped for HPV using PCR. HPV infection, defined as p16 positivity and a positive HPV DNA status, was identified in 14% of the cohort. The majority (83%) of the HPV-positive tumours involved the oropharynx while the oral cavity, pharynx and the nasal cavity (17%) were involved to a much smaller extent. High-risk HPV type 16 was the most prevalent HPV type. Patients with HPV-positive tumours had significantly improved overall survival (OS) (2 year OS, 77% vs 57%) and recurrence free survival rates (RFS) (2 year RFS, 92% vs 77%) compared to patients with HPV-negative tumours. A positive tumour HPV status was found to be an independent prognostic indicator (HR 0.216; 95% CI 0.06 – 0.771) and so, given the high morbidity and debilitating physical and psychological problems associated with prevailing aggressive treatment regimens, it is imperative that this knowledge is harnessed to develop and improve treatment strategies.

EBP50 expression was evaluated, by immunohistochemical analysis, first in normal oral mucosa and followed up in a smaller subset of 156 HNSCC patients from the main cohort. In the normal tissue EBP50 expression was predominantly membranous. In the tumour samples four distinct EBP50 expression patterns were observed and, of these,

weak/ negligible cytoplasmic EBP50 expression showed a strong correlation, only marginally lower than p16 overexpression, with HPV DNA status and was observed largely in patients with tumours of the oropharynx and no history of smoking. Absence of EBP50 expression in the plasma membranes of tumour cells was a recurring pattern in a majority of the tumour samples.

The scale of this study, comprising a Tayside cohort of unprecedented size, will undoubtedly contribute to the existing knowledge of HPV incidence in head and neck cancer in Scotland. Furthermore, this study presents compelling preliminary evidence for further researching weak/negligible cytoplasmic EBP50 expression as being a potential indicator of HPV-positivity in HNSCC.

Chapter 1

INTRODUCTION

1.1 Squamous cell carcinomas of the head and neck

Head and neck squamous cell carcinomas (HNSCC) are a heterogeneous group of tumours arising in the moist mucosal surfaces lining the upper aero-digestive tract and constitute the majority of head and neck malignancies [107]. Depending on the specific head and neck subsite/area in which they originate, primary tumours are categorised into those arising in the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, paranasal sinuses and nasal cavity and the salivary glands (Figure 1). An estimated 600,000 new cases of HNSCC are reported worldwide each year with the most common sites being the oral cavity, the larynx and the pharynx [196], [26].

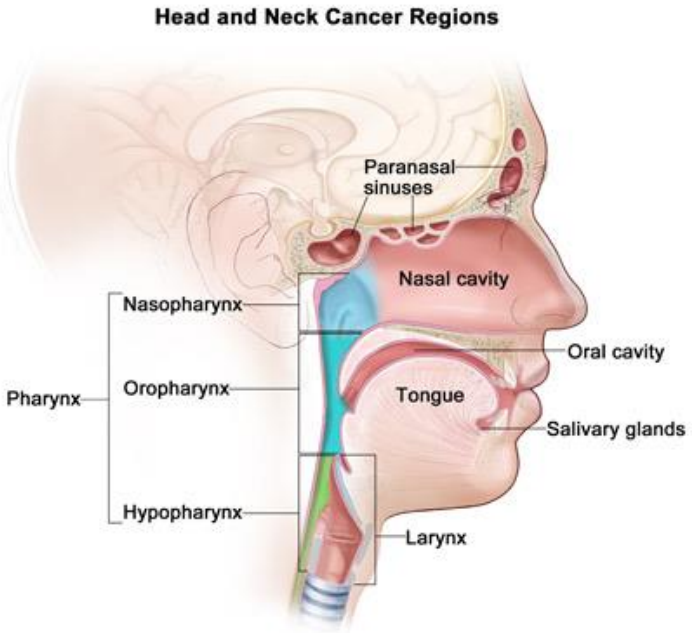

Figure 1.1 (National Cancer Institute) Illustration of head and neck subsites.

1.1.1 Incidence of HNSCC in the UK and Worldwide

HNSCCs account for approximately 4% of all malignancies and 5% of cancer related mortality worldwide with considerable variation in incidence rates depending on geographic location and gender. Globally, the highest incidence rates for HNSCC, particularly cancers of the oral cavity and oropharynx, have been reported in South and Southeast Asia (e.g. Sri Lanka, India, Pakistan and Taiwan), parts of Western Europe (e.g. France) and Eastern Europe (e.g. Hungary, Slovakia and Slovenia), parts of Latin

America and the Caribbean (e.g. Brazil, Uruguay and Puerto Rico) and in the Pacific regions (e.g. Papua New Guinea and Melanesia) [316]. Incidence rates are generally more than two-times higher in men compared to women, although incidence rates up to ten-fold higher have been reported in the Republic of Slovakia and Belarus [280].
In the United Kingdom, oral and pharyngeal cancers accounted for 2% of all new cancers diagnosed in 2013 [34]. According to Cancer Research UK, there are 14 new cases of oral cancer for every 100,000 males and 7 new cases for every 100,000 females [34]. Incidence rates are higher in Scotland than other parts of the UK for both men and women [50]. Surprisingly, a rising incidence of oral cancers in young people under the age of 45 years has been recorded by UK cancer registries [316].

1.1.2 Aetiology of HNSCC

Major risk factors for HNSCC are alcohol consumption and tobacco use and they account for nearly three-quarters of all head and neck cancers. While tobacco and alcohol use independently increase the risk of head and neck cancer, cigarette smoking is more strongly associated with an increased risk, especially, of laryngeal cancer [113]. The chewing of areca nut and betel quid (with or without tobacco) are additional risk factors specific to South Asian countries where the use of these products is widespread[86].

As is the case with many other diseases, the risk of head and neck cancer increases when an individual is predisposed to it as a result of specific genetic variations which are often inherited. For example, individuals with deletion polymorphisms in the genes GSTM1 and GSTT1 that encode for the Glutathione S Transferase family of enzymes responsible for metabolising and detoxifying carcinogens found in tobacco smoke, were found to be at an increased risk of developing head and neck cancer [230]. In yet another example, pooled analyses of epidemiological studies of risks associated with head and neck cancer found that a family history of the disease increased the likelihood of the close blood relatives developing the disease [50].

Infectious agents, predominantly viruses, have been recognised as a risk factor for head and neck cancer - the earliest evidence for which is the association between nasopharyngeal cancer and infection with the Epstein-Barr virus. In the recent years, infection with high-risk Human papillomavirus (HPV) has emerged as a strong risk

factor for the development of head and neck cancer, especially that of the oropharynx [196].

Social deprivation has been linked to HNSCC risk in high and low income countries across the world. While the reasons for this are poorly understood, it has been suggested that the low levels of income and education associated with social deprivation may affect nutrition, living conditions and access to health care and also influence risky health behaviour patterns [51], [303]. Other risk factors include occupational exposure, poor oral hygiene and reduced intake of fruit and vegetables [196].

1.1.3 Clinical signs and symptoms

Patients with HNSCC present with a variety of symptoms, some of which are specific and depend on the function of the tumour site of origin while others are relatively non-specific. Patients with cancers of the oral cavity often present with non-healing mouth ulcers whereas a common presentation of laryngeal cancer is hoarseness of voice. Many patients often present with painless swelling in the neck and non-specific symptoms such as persistent sore throat or ear pain [196]. Practice guidelines outlined by the National Institute of Clinical Excellence (NICE) recommend urgent referral and specialist consultation for patients presenting with certain signs and symptoms that persist for more than three weeks (Table 1.1) [225].

Symptoms	Signs
• Sore throat • Hoarseness • Difficulty in swallowing/breathing • Lump in the neck • Unilateral ear pain	• Red or white patch in the mouth • Oral ulceration • Mobile tooth • Lateral neck mass • Rapidly growing thyroid mass • Cranial nerve palsy • Orbital mass • Unilateral ear effusion

Table 1.1 "Red Flag" symptoms and signs in head and neck cancer [225]

1.1.4 Diagnosis and treatment

In a specialist head and neck clinic, patients undergo clinical examination followed by a series of diagnostic tests. Suspicious lesions are biopsied and sent for histological examination which often confirms a diagnosis of cancer at the primary site. Additionally, computed tomography and magnetic resonance imaging techniques may be used to determine the extent of the primary tumour. Nodal metastasis may be investigated through ultrasound guided fine needle aspiration cytology and occult primaries and distant metastasis are investigated through Positron Emission Tomography [196].

Although the contribution of HNSCC to global cancer burden is relatively small, it is associated with high morbidity, high mortality due to the aggressive nature of the disease, and limited therapeutic options, therefore making early diagnosis and treatment paramount. Prognosis for patients with HNSCC is dependent on several factors, the most important ones being the site and stage of the disease. Head and neck cancer staging is based on the universally accepted TNM classification system of the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC). The TNM system describes the extent of the primary tumour (T), presence and extent of regional lymph node metastasis (N), and presence or absence of distant metastasis (M). This classification may be assigned before or after histopathologic examination of the biopsied/resected primary tumour and is referred to as clinical or pathologic TNM classification respectively [298]. The TNM classifications for the most common head and neck cancer sites are described in Table 1.2. Table 1.3 is a summary of HNSCC staging based on TNM classifications.

Site	Primary tumour (T)	Regional lymph nodes (N)	Distant metastasis (M)
	TX - Primary tumour cannot be assessed **T0** - No evidence of primary tumour **Tis** - Carcinoma *in situ*	**NX** - Regional nodes cannot be assessed **N0** - No regional lymph node metastasis	**M0** - No distant metastasis **M1** - Distant metastasis
Oral cavity	**T1** - Tumour≤ 2cm **T2** - Tumour > 2cm but < 4cm **T3** - Tumour > 4cm **T4a** - Tumour invades through cortical bone, deep/extrinsic muscle of tongue, maxillary sinus, skin **T4b** - Tumour invades through masticator space, pterygoid plates, skull base, internal carotid artery	**N1** - Metastasis in a single ipsilateral lymph node ≤ 3 cm **N2** - Metastasis in a single ipsilateral lymph node > 3 cm but < 6 cm; or in multiple ipsilateral lymph nodes, none > 6 cm; or in bilateral or contralateral lymph nodes, none > 6 cm **N2a** - Metastasis in a single ipsilateral lymph node > 3 cm but not more than 6 cm **N2b** - Metastasis in multiple ipsilateral lymph nodes, none > 6 cm **N2c** - Metastasis in bilateral or contralateral lymph nodes, none > 6 cm **N3** - Metastasis in a lymph node > 6 cm	
Oropharynx	**T1** - Tumour≤ 2cm **T2** - Tumour > 2cm but < 4cm **T3** - > 4cm or extension to lingual surface of the epiglottis **T4a** - Tumour invades the larynx, deep/extrinsic muscle of the tongue, medial pterygoid, hard palate, or mandible **T4b** - Tumour invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases the carotid artery		
Hypopharynx	**T1** - Tumour ≤ 2 cm and/or limited to one subsite **T2** - Tumour >2 cm but < 4cm or invades more than one subsite **T3** - > 4 cm or with hemilarynx fixation **T4a** - Tumour invades thyroid/cricoid cartilage, hyoid bone, thyroid gland, or central compartment soft tissue **T4b** - Tumour invades		

	prevertebral fascia, encases carotid artery, or involves mediastinal structures		
Larynx - Supraglottis	**T1** - Tumour limited to 1 subsite of the supraglottis with normal vocal cord mobility **T2** - Tumour invades mucosa of more than 1 adjacent subsite of the supraglottis or glottis or region outside the supraglottis **T3** - Tumour limited to the larynx with vocal cord fixation and/or invades any of the following: postcricoid area, pre-epiglottic space, paraglottic space, and/or inner cortex of the thyroid cartilage **T4a** - Tumour invades through the thyroid cartilage and/or invades tissues beyond the larynx T4b - Tumour invades prevertebral space, encases carotid artery, or invades mediastinal structures		
Larynx-Glottis	**T1** - Tumour limited to the vocal cord(s) (may involve anterior or posterior commissure) with normal mobility **T1a** - Tumour limited to 1 vocal cord **T1b** - Tumour involves both vocal cords **T2** - Tumour extends to the supraglottis and/or subglottis, and/or with impaired vocal cord mobility **T3** - Tumour limited to the larynx with vocal cord fixation and/or invasion of the paraglottic space, and/or inner cortex of the thyroid cartilage **T4a** - Tumour invades through the outer cortex of the thyroid cartilage and/or invades tissues beyond the larynx T4b - Tumour invades		

	prevertebral space, encases carotid artery, or invades mediastinal structures		
Larynx-Subglottis	**T1** - Tumour limited to the subglottis **T2** - Tumour extends to vocal cord(s) with normal or impaired mobility **T3**- Tumour limited to the larynx with vocal cord fixation **T4a** - Tumour invades cricoids or thyroid cartilage and/or invades tissues beyond the larynx **T4b** - Tumour invades prevertebral space, encases carotid artery, or invades mediastinal structures		

Table 1.2 TNM classification for primary tumours of the oral cavity, oropharynx, hypopharynx and larynx [243]

UICC stage	Primary tumour (T)	Regional lymph node (N)	Distant metastasis (M)
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T Any	N3	M0
	T4b	N Any	M0
IVC	T Any	N Any	M1

Table 1.3 Head and neck cancer staging [243]

Patients with tumours involving metastases to the regional lymph nodes and other tissues have poorer survival [299], and comorbid illnesses can also significantly affect patient survival [233]. In addition, certain histopathological features such as the degree of tumour differentiation, tumour invasive pattern and perineural invasion and extracapsular spread are reliable indicators of prognosis in patients with head and neck cancer [274], [293], [76]. Although a few molecular markers such as epidermal growth factor, tumour hypoxia associated markers and vascular endothelial growth factors have been studied and reported to be associated with poor prognosis, none have been included in routine clinical reporting [197].

Given the complex nature of the disease, patients with head and neck cancer are often treated by multidisciplinary teams to ensure that they receive a high standard of care. Surgery and radiotherapy are the two most common treatment options and the choice of treatment depends on factors related to the site and stage of the tumour as well as patient preference [197]. About one-third of the patients present with early stage disease and are treated with surgery or radiotherapy with favourable prognosis. The majority, however, present with locally advanced tumours and are treated with a combination of surgery and post-operative chemotherapy and radiotherapy. Over the last decade, the standard of care has shifted from aggressive treatment protocols to organ preserving multi-modal therapy that combines treatment with systemic chemotherapeutic drugs and loco-regional radiation. The very advanced hypopharyngeal and laryngeal carcinomas are treated by surgery followed by post-operative (chemo) radiation. Little has changed in the overall 5-year survival for the advanced disease in decades because patients frequently develop relapse at the primary site, distant metastases and second primary tumours [27].

Treatment regimens for head and neck cancer are associated with early and long term complications which are often severely debilitating, thereby greatly affecting the patient’s quality of life [228]. To ensure tumour clearance, surgery may require the sacrifice of important neuromuscular structures such as the sternocleidomastoid muscle which causes loss of contour to the neck and, occasionally, injury to the spinal accessory nerve during surgery leads to shoulder dysfunction [149]. Radiotherapy is associated with serious late toxicities such as xerostomia, osteo-radionecrosis and dysphagia [108]. Survivors of head and neck cancer present with functional problems such as dry mouth, difficulty in swallowing, speech defects and weak shoulders all of

which significantly impair long term quality of life [180]. Less aggressive treatment protocols with a focus on personalised care based on diagnostic and prognostic markers could reduce morbidity in this patient population.

1.1.5 Screening and prevention

Despite recent advances in treatment options, HNSCC is associated with significant morbidity and mortality and the costs related to management of the disease are high [325]. Part of the problem may be attributed to the lack of screening programmes for HNSCC and that patients often present with locally advanced disease. Early detection through screening programmes has certainly proven to be effective in reducing mortality and incidence rates of other major cancers such as breast and cervical cancers [67], [52]. There are no national population based screening programmes available for HNSCC. However, a few instances of opportunistic visual screening in high-risk groups in areas of high incidence have been reported [262]. Although adjunctive methods such as toluidine blue, brush biopsy and fluorescence imaging are available for detection of precancerous lesions, there is no evidence to support the efficacy of these methods in the general population, and furthermore, such methods may not be productive for detecting precursor lesions in clinically inaccessible head and neck subsites. The net result of all this is that, currently, clinical examination and biopsy form the mainstay of HNSCC screening [153].

Lifestyle risk factors and negative health behaviours associated with HNSCC are linked to public knowledge of this disease and can influence early detection. Public awareness of head and neck cancer in the UK is limited, although local initiatives are being set up. A qualitative study conducted by Scott *et al*. found that a high-risk target population had limited knowledge about oral cancer (particularly signs and symptoms) and also found that participants were uncertain about when to seek help for oral symptoms [270]. Efforts to raise awareness among the public and healthcare professionals alike by developing strategies to change lifestyle risk factors and to detect early signs and symptoms may decrease the disease burden of HNSCC.

1.2 Changing epidemiology of head and neck squamous cell carcinomas

The use of tobacco remains an undisputed risk factor for the development of HNSCC.

In the United States, mounting evidence against the hazards of tobacco use and its association with various diseases has resulted in decreased consumption since the mid 1960s and it was anticipated that the incidence of HNSCC would decline with a decrease in tobacco use [6]. Indeed, the incidence rates of carcinoma of the larynx, floor of the mouth and other oral cavity sites have declined steadily since 1980s in the US, the UK and Europe [37]. On the contrary, a small subset of HNSCC, the oropharyngeal squamous cell carcinomas (OPSCC) which involves the tonsils, base of tongue, soft palate and pharyngeal wall has been rising rapidly and steadily over the last decade in the US, the UK, Sweden, Greece and Australia [6], [194]. A compelling body of evidence points to infection with high-risk Human Papillomavirus (HPV) as the main contributor in this changing epidemiological trend [111], [189].

1.3 Human Papillomavirus and cancer

Viruses have had a long-standing association with cancers and are linked to an estimated 15-20 percent of all human cancers worldwide, representing a significant portion of the global cancer burden [331]. Since the discovery, in the 1950s, of the Epstein-Barr virus in Burkitt's Lymphoma cells [60], marking the emergence of the first conclusive evidence of the role of viruses in the pathogenesis of human cancers, several distinct groups of viral pathogens carcinogenic to humans have been identified - Hepatitis B and C viruses (liver cancer), Human Papillomaviruses (cervical, anogenital and oropharyngeal cancers), Herpesvirus (Kaposi's sarcoma), and human T-cell lymphotropic virus (adult T-cell leukemia), among others [24] .

Not all viral infections progress to cancer and, in fact, viral carcinogenesis is a result of several years, sometimes decades, of chronic inflammation complicated by additional events and host factors such as host genetic predisposition, somatic mutations, immune suppression and exposure to carcinogens. Both DNA and RNA viruses have been shown to be capable of causing cancer in humans [171]. Of the DNA viruses with a malignant potential, Hepatitis B virus, Human Herpes Virus 8, Epstein Barr Virus and HPV have been widely studied [171].

Human Papillomavirus is a member of the papillomaviridae family which comprises a diverse group of more than one hundred and fifty related viruses with different epithelial tropisms and life cycle strategies [61]. These viruses are classified, based on

their nucleotide sequences, into 5 groups: the Alpha, Beta, Gamma, Nu and Mu papillomaviruses [61]. The Alpha papillomaviruses constitute the largest group and are further subdivided into high-risk and low-risk depending on their prevalence in the general population and their carcinogenic potential [64]. The low-risk HPV types are rarely ever associated with cancer development; however, they are known to cause recurrent and debilitating disease conditions such as recurrent respiratory papillomatosis in some individuals which may eventually conclude in metastatic tumours of the lower respiratory tract [64], [96].

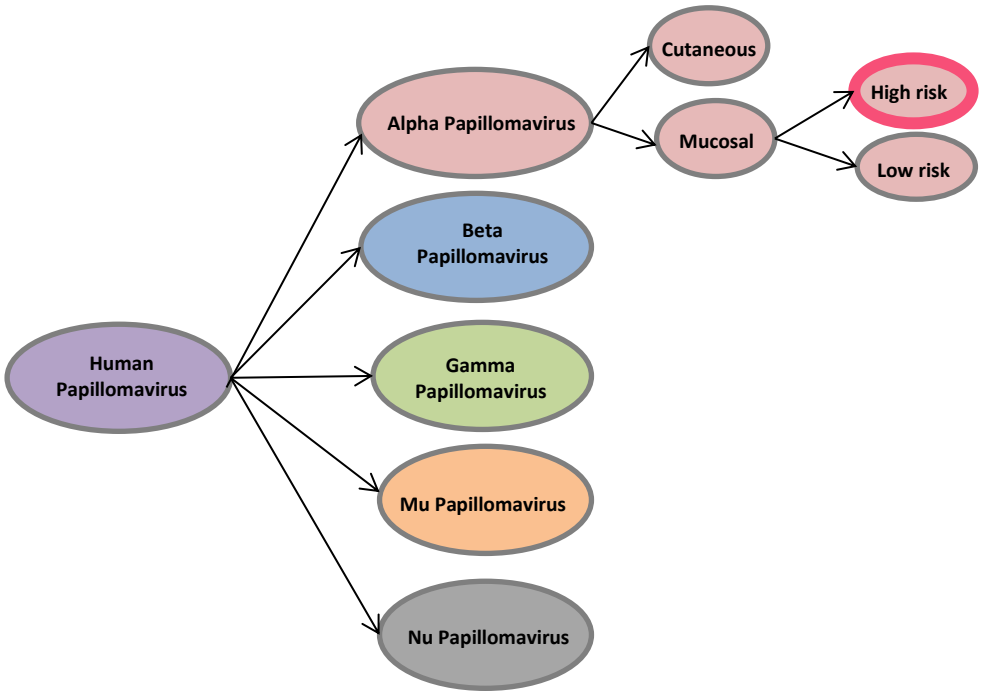

Figure 1.2 Classification of Human Papillomaviruses. They are divided into 5 groups based on their nucleotide sequences; the Alpha, Beta, Gamma, Nu and Mu papillomaviruses. The Alpha papillomaviruses constitute the largest group and are further subdivided into high-risk and low-risk types. The high-risk HPVs are of particular aetiological importance in the development of cervical, anogenital and head and neck cancer.

The global incidence of HPV-related cancers has been on the rise over the last decade and is now a major worldwide public health concern [236]. The WHO currently recognises twelve HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) as high-risk cancer causing types and types 68, 73 as 'possibly' cancer causing [24]. The high-risk HPV types 16 and 18 are responsible for the majority of HPV-related cancers [24]. In fact, HPV type 16 is responsible for almost 99.9% of cervical cancer and cervical intraepithelial neoplasia grade 3 worldwide [314]. HPVs are also implicated in a variable proportion of non-cervical neoplasms including anal (90%), vaginal (40%) and penile (40%) [8].

In the upper aerodigestive tract, HPV has been linked to cancers of the oral cavity, oropharynx and the larynx. A systematic review conducted by Ndiaye *et al*. showed that 45.8% of OPSCC, 24.2% of oral cavity and 22% of all laryngeal cancers were attributed to HPV [219]. While the role of HPV in the development of oral cavity and laryngeal cancers is contentious, its attribution to OPSCC is certain [119], [178]. There has been substantial variability in the reported worldwide prevalence of HPV-associated HNSCC ranging from 16%-46% [148]. This geographic heterogeneity may be due to small study samples, sample selection and variability in classification of head and neck subsites [148].

While cancers attributable to HPV are more common in women compared to men, owing to the large burden of cervical cancer, this gender disparity varies significantly with geographic distribution and economic development of the country. The Population Attributable Fraction (PAF) of HPV in women ranged from less than 2.5% in North America and Australia to 25% in India and sub-Saharan Africa and that of men was highest in India (1.8%) [102]. The lack of robust cervical screening programmes leading to a higher incidence in cervical cancer coupled with lower incidence in HPV-related OPSCC might explain the gender disparity observed in less developed countries. On the other hand, industrialised countries have witnessed a decline in the incidence of, and mortality from, cervical cancer due to the introduction of successful cervical screening programmes [6]. However, this decline is offset by an increase in the incidence of HPV-related non-cervical cancers especially, oropharyngeal cancers [194], [181].

1.3.1 Molecular mechanisms underlying HPV-driven tumourigenesis – a cervical cancer model

Our understanding of the natural history of HPV infection is gleaned from cervical cancer studies. Cervical cancer which arises from the cervical transformation zone is a complex, typically progressive series of events beginning with transmission of viral infection followed by viral persistence, progression of a group of abnormal cells to pre-cancer and culminating in invasion.

HPV infections are primarily sexually transmitted via direct skin or mucosa contact and, as such. are common in young sexually active individuals [65]. The majority of these infections are cleared spontaneously in 12 -24 months through effective cell-mediated

immune responses in the host. However, a small fraction of infections persist for several years and carry with them an increased risk of pre-cancer diagnosis [268], [267]. The time-to-appearance of pre-cancer following infection with the virus can be as short as 3-5 years with highly carcinogenic HPV-16 [266]. Progression to invasive carcinoma due to persistent infection is believed to be partly due to an inability of the host to develop an effective cell-mediated immune response and is exacerbated in combination by immune evasion strategies developed by the virus [65].

The HPV genome comprises a circular double stranded DNA molecule of 8000 base pairs organised into three regions: a 4000 bp region that encodes proteins involved in cell transformation and viral replication, a 3000 bp region which encodes structural proteins of the virus and a 1000 bp region which contains the origin of the viral DNA replication and transcriptional regulatory elements [210]. There are two clusters of genes that constitute the viral genome; the early genes E1, E2, E4, E5, E6 and E7 and the late genes L1 and L2 (Figure 1.3). The early genes E1 and E2 encode regulatory proteins which are essential for DNA replication. E4 and E5 also contribute to viral genome amplification although E4 appears to be expressed in the late stages of viral infection. The viral genes E6 and E7 encode oncoproteins responsible for host cell transformation. The late genes L1 and L2 encode two structural capsid proteins which are utilised in the construction and assembly of new viral particles [248].

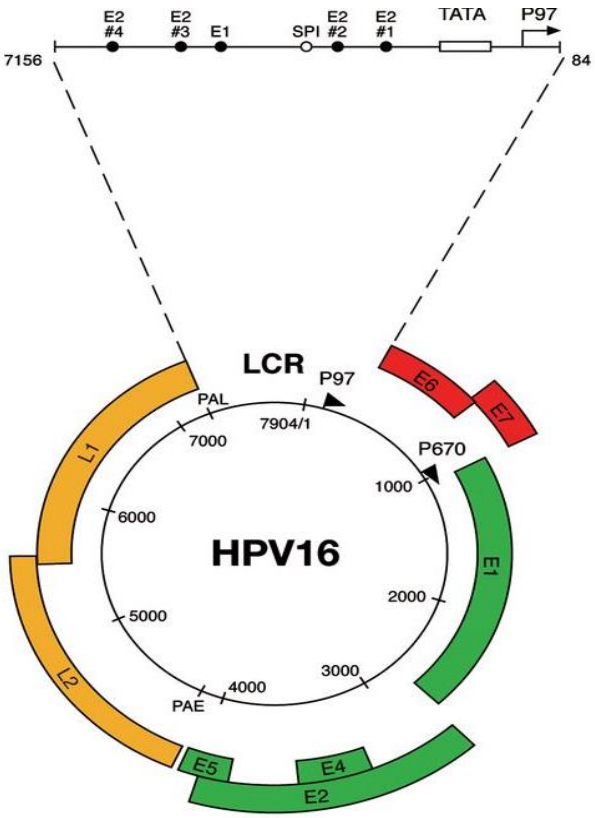

Figure 1.3 The HPV-16 genome comprises 7904 bp (shown in black). The early genes E1, E2, E4 (green), E6 and E7 (red) are expressed from either the early promoter p97 or the late promoter p670 (marked as black arrows). The late genes L1 and L2 (yellow) are also expressed from p670. The LCR (long control region) extends from 7156 – 7184 bp and harbours E2 binding sites and the TATA binding element of the p97 promoter [64].

The HPV life cycle is closely linked to the host epithelial cell differentiation pattern. Normal stratified squamous epithelium has a basal layer of slow-cycling, self-renewing population of cells that are ideal for maintenance of the virus. The virus enters the tissue through wounds or epithelial trauma and infects the epithelial basal stem cells. The cervical transformation zone is especially susceptible to infection and cancer progression owing to the presence metaplastic cells at the site, particularly at puberty and the presence of cuboidal stem-like cells at the squamo-columnar junction [106], [118]. The precise mode of viral entry is unclear, but interactions with heparin proteoglycans and α6 integrin have been suggested [239], [193]. Following infection of the basal cells, the HPV early genes E1 and E2 support the synthesis of low copy numbers of episomal genomes through viral DNA replication (Table 1.4). As the infected cells differentiate and migrate to the supra-basal layers of the epithelium, viral late genes products initiate the vegetative phase of the HPV lifecycle, resulting high-level amplification of the viral genome. Viral DNA is encased in capsid proteins in the outer epithelial layers and progeny virions are released to initiate infection once again. Although the progeny virions are highly immunogenic, they encounter limited immune

surveillance as they are synthesised in the upper layers of the epithelium [217]. In addition, the E6 and E7 proteins affect the host immune response by interfering with JAK/STAT (Janus kinase/signal transducers and activators of transcription) signalling, induction of Interferon Response Factor-1 (IRF-1) and reducing the levels of MHC-1 (Major Histocompatibility Complex) antigen presentation [169], [307]. Such immune evasion mechanisms allow the virus to remain as a chronic asymptomatic infection.

Early genes	***Function***
E1	Initiation of viral genome replication
E2	Viral DNA replication and transcription
E4	Packing of viral particles
E5	Host cell transformation. Inhibition of apoptosis in late events of HPV carcinogenesis.
E6	Inactivates p53 protein and blocks apoptosis. Interacts with many host proteins with PDZ domains, (PSD-95 - a 95 kDa protein involved in signalling at the post-synaptic density, DLG - the Drosophila melanogaster Discs Large protein and ZO-1 the zonula occludens 1 protein) involved in maintenance of epithelial polarity.
E7	Inactivates pRb protein. Promotes host DNA synthesis and proliferation.
Late genes	***Function***
L1	Major capsid protein
L2	Minor capsid protein

Table 1.4 Early and late genes of the HPV genome and their functions [248].

The carcinogenic potential of high-risk HPVs may be attributed to the viral oncoproteins E6 and E7. Integration of viral DNA into the host cell genome appears to be an important step in the malignant transformation of host tissue. Integrated viral DNA has a greater potential for neoplastic transformation by initiating a series of events which lead to overexpression of the E6 and E7 oncoproteins [64]. The E6 and E7 oncoproteins in most HPV types stimulate host cells in mid-epithelial layers to re-enter the cell cycle, thereby facilitating viral genome amplification [65]. Furthermore, they promote carcinogenesis in the infected tissues by targeting and disrupting multiple cellular pathways (Table 1.4).

Both E6 and E7 are necessary to induce and maintain cellular transformation. The most important function of the E6 protein in cellular transformation is to support the accumulation of mutations by disrupting p53 mediated DNA repair. The p53 tumour

suppressor protein plays an important role in the induction of cell cycle arrest and apoptosis following DNA damage. Moderate DNA damage is countered by a p53 dependent cell cycle arrest followed by DNA repair but when the damage is severe, apoptosis is induced [217]. The E6 protein combines with p53 and cellular ubiquitin ligase E6 associated protein (E6AP) to form a tripartite complex which leads to proteasome mediated degradation of p53. As a result, cells expressing high-risk HPV E6 are no longer able to counter DNA damage due to the loss of p53 leading to the accumulation of secondary changes in the host cell genome that eventually progress to cancer [64]. In addition, E6 interferes with pro-apoptotic proteins such as Bak, Fas associated death domain (FADD) and procaspase 8 to further prevent apoptosis [301], [92]. Another important function of the E6 protein is its ability to induce telomerase activity - whose absence in normal cells implies the natural shortening of telomeres with each cell division and eventually to cell senescence and death - by activating its catalytic subunit human telomerase reverse transcriptase (hTERT) [138]. Due to induced telomerase activity, E6 infected cells are able to maintain telomere length leading to immortalisation [138]. High-risk E6 proteins also target and degrade PDZ domain containing proteins such as human homologue of the *Drosophila* disc large protein (hDlg) and human homologue of the *Drosophila* scribble protein (hSrib) [90], [214]. Specifically, PDZ proteins play an important role in cell polarity, signalling and cell adhesion and high-risk E6 proteins bind to these proteins through their C-terminal PDZ binding domain (PBM) and stimulate their degradation. E6 induced degradation of PDZ domain proteins is thought to result in the loss of cell polarity seen in HPV-induced cervical cancers owing to a disruption of tight junctions [191].

The most important function of the high-risk E7 protein is to promote cellular proliferation by binding to, and, inactivating the Retinoblastoma protein (pRB) and its family members p107 and p130. The pRB proteins, in their hypophosphorylated state, bind to transcription factor E2F to supress the transcription of genes involved in DNA synthesis and cell-cycle progression. Phosphorylation of pRb by G1 cyclin-dependent kinases releases E2F leading to cell cycle progression into the S phase. E7 is capable of binding to unphosphorylated pRB and, in doing so, may force the cells to enter S phase prematurely by disrupting pRB-E2F complexes. Another consequence of E7 mediated degradation of pRB proteins is the overexpression of cyclin dependent kinase inhibitor p16 INK4a (p16). p16 is a tumour suppressor that prevents phosphorylation of pRB. However, disruption of the pRB-E2F complex can cause the transcriptional factor E2F

to induce p16 INK4a expression. p16 overexpression is thus controlled by the functional status of pRB and E7 mediated inactivation of pRB can result in its overexpression [129].

Other transforming properties of E7 include association with histone deacetylases (HDAC) to promote cell growth and cyclin dependent kinase (CDK) inhibitors p27 and p21 to abrogate cell-cycle inhibition [217]. Table 1.5 summarises all the important cell processes affected by the E6 and E7 proteins [202].

Viral oncoprotein	Transforming property	Biological impact
E6	Degradation of tumour suppressor protein p53	Accumulation of cellular abnormalities increasing the potential for malignant transformation
E6	Impairment of p53-induced gene transcription by binding to transcriptional co-activators CREB binding protein (CBP) and p300	Interference with cell differentiation and cell cycle progression
E6	Degradation of cell polarity and PDZ domain proteins	Loss of cell-cell adhesion increasing the invasive potential of transformed cells
E6	Degradation of pro-apoptotic protein Bak	Inhibition of cell death and accumulation of abnormal cells
E6	Increased transcription of Human Telomerase Reverse Transcriptase (hTERT)	Immortalisation of affected cells and indefinite proliferation
E7	Inactivation of tumour suppressor protein pRb	Disruptions of cell cycle check points. Increased cell proliferation
E7	Binding to cyclin-dependent-kinase (CDK) inhibitors p21 and p27	Disruption of cell cycle and increased cell proliferation
E6 and E7	Inhibition of expression of toll-like receptor 9 (TLR9)	Evasion of immune surveillance

Table 1.5 The biological impact of HPV transforming proteins E6 and E7. Both E6 and E7 promote and maintain cellular transformation. The E6 protein supports the accumulation of mutations by disrupting p53 mediated DNA repair. The E7 protein promotes cellular proliferation by inactivating pRB. In addition, the E6 and E7 proteins interfere with the functions of several other cellular proteins.

1.4 HPV and HNSCC

HPV-induced carcinogenesis in the upper aerodigestive tract is less well understood in comparison to cervical cancers. Oral HPV infection is a supposed precursor of HPV-related OPSCC and is strongly associated with sexual behaviour [55]. The rising

incidence of HPV-positive OPSCC in the USA has been attributed to changes in sexual norms such as lower age of first sexual encounter and higher numbers of lifetime sexual partners per individual [100]. The natural history of oral HPV infection, its clearance and persistence are all crucial to understanding how oral HPV infection progresses to malignancy. Attempts to study the progression of oral HPV infection to malignant cancer have been hampered by the lack of robust screening methods and a general absence of pre-malignant lesions in the head and neck regions as compared to cervical intraepithelial neoplasia (CIN) [59].

1.4.1 Oral HPV infection

The overall prevalence of oral HPV in the general population has been estimated to be between 1.8 – 7.5% [160]. In a pooled analysis, Kreimer *et al.* reported the prevalence of oral HPV infection in healthy adults to be 4.5% and the prevalence of oncogenic HPV-16 to be 1.3% [147]. In a recent large-scale, multi-centre study conducted across ten European countries including the UK, HPV-16 L1 antibodies were detected in 2.6% of the healthy cancer-free subjects. Additionally, this study also found that HPV-16 seropositivity was associated with an increased risk of oral cavity and oropharyngeal cancer [30].

Identifying risk factors for oral HPV infection is not only important in understanding the natural history of the disease but also in identifying high-risk populations. Independent risk factors which have been identified for oral HPV infection include gender, age, tobacco use, high-risk sexual behaviour and immunosuppression [160]. Oral HPV infection has been shown to be significantly higher in men than in women [56] and differences in risk behaviours and immune response to viral acquisition and clearance influenced by sex hormones may account for this [160]. While increasing age has been significantly associated with oral HPV infection, probably owing to age related loss of immunity, a study by Gillison *et al.* reported a bimodal pattern where prevalence peaked among individuals aged 30 to 34 years and 60 to 64 years [99]. With respect to tobacco use, higher oral HPV prevalence was found in current tobacco users compared to ex-smokers [77] and those who never smoked. Additionally, smoking appears to increase the risk of persistence of oncogenic HPV infection which is crucial for malignant transformation [133]. Given that the primary mode of transmission of HPV infection is sexual contact, evidence suggests that certain high-risk sexual behaviour patterns are associated with transmission of oral HPV infection and

subsequent malignant transformation. A large scale multi-center study conducted by The Head and Neck Epidemiology Consortium (INHANCE) reported an increased risk of oropharyngeal cancers for individuals with a history of six or more lifetime sexual partners, four or more lifetime oral sexual partners, early age at sexual debut and a history of same-sex sexual contact among men [116]. The higher incidence and prevalence of oral HPV infection and OPSCC seen in HIV-affected individuals indicates that oral HPV is strongly associated with immunosuppression [12].

1.4.2 HPV-driven tumourigenesis in the upper aerodigestive tract

HNSCC cancers are most commonly associated with high-risk HPV-16 which is perhaps the most aggressive type. This may be explained in part by the high prevalence of HPV-16 in the general population and evolution of the oral epithelium making it accessible only to the most virulent HPVs. Evidence exists to show that unlike cervical cancers, DNA integration is not necessary for the development of head and neck cancers and that the virus can exist in episomal forms. For example, Koskinen *et al*. reported that in their cohort of head and neck cancers, 61% were HPV DNA positive. HPV-16 was the dominant sub-type, and found in 84% of HPV-positive cancers. Real time (RT-PCR) analysis demonstrated that of the HPV-16-positive samples, 48% were integrated, 35% were episomal and 17% were mixed with both episomal and integrated forms [141].

Viral particle internalisation into target cells is thought to be initiated by binding to cell surface heparan-sulfonated proteoglycans (HSPGs) and also by interacting with secondary receptors [65]. There is evidence to suggest that during the process of infection, HPV activates growth factor receptors triggering the PI3K/Akt/mTOR (Phosphoinositide 3-kinase/ serine-threonine protein kinase B/mammalian Target Of Rapamycin) signalling pathway [292]. The PI3K pathway has been implicated in multiple human cancers and is activated when Epidermal Growth Factor Receptor (EGFR) binds to its ligand activates leading to the phosphorylation of phosphatidylinositol on the 3'- hydroxyl group. The product, phosphatidylinositol-3, 4, 5-triphosphate activates AKT kinase which in turn phosphorylates many downstream proteins that regulate cell growth and survival [66]. A study conducted by Surviladze *et al*. showed that the Akt/mTOR signalling pathway was rapidly activated when human keratinocytes were exposed to HPV type 16 pseudovirions (PsVs) suggesting that HPV infection stimulates the PI3K/Akt/mTOR pathway via α-6 β -4 integrins [292]. HPV

oncoprotein expression confers a very distinct molecular profile on HNSCC. For example, HPV-positive HNSCCs show lower rates of p53 and p16INK4a alterations when compared to their negative counterpart (Table 1.6) [29]. Additionally, viral E6/E7 oncoprotein expression in various anatomic sites in the head and neck will often control specific cell signalling pathways. A few studies have provided valuable insight into the role HPV E6 expression in the activation and manipulation of the Wnt cell signalling pathway which is essential in regulation of proliferation and differentiation responses during normal development. Activation of the Wnt signalling pathway inhibits GSK-3β which leads to accumulation of cytoplasmic β- catenin and its translocation into the nucleus where it binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and induces expression of numerous proto-oncogenes, including c-*myc* and *cyclin D1*, as well as genes which regulate growth and tumour progression, such as *MMP7, PPARδ, gastrin, connexin 43,* and WISP proteins [33]. In a study conducted by Smeets *et al.* the authors showed that immortalisation of normal oral keratinocytes (OKCs) by HPV E6 expression caused an activation of Wnt pathway which was consistent with the finding that nuclear β - catenin protein levels were upregulated in HNSCCs and HPV16-positive oropharyngeal cancer cell lines [284].

1.4.3 Implications of high-risk HPV-driven tumourigenesis in the upper aerodigestive tract

Over the past decade, one of the most significant developments in the field of head and neck oncology is the establishment of the prevalence of HPV in a small subset of HNSCC. The incidence of HPV-associated HNSCC has risen steadily over the last 40 years [43], [189], [218] and is set to escalate over the next twenty years [246]. Patients with HPV-positive HNSCC represent a demographically and clinically distinct patient population with improved prognosis [189]. Furthermore, HPV-positive tumours have specific histopathological features [324]. The rising incidence of HPV-related HNSCC has several clinical implications with patient survival being, perhaps, the most important one.

1.4.3.1 Demographic, clinical and histopathological characteristics of patients with HPV-related HNSCC

Demographically, patients afflicted with HPV-related HNSCC are more likely to be younger or middle aged men with little or no history of smoking and alcohol consumption (Table 1.6) [6]. The role of smoking in HPV-related HNSCC is debatable.

While some studies have shown that HPV-positive OPSCC patients are never smokers, others have reported no significant differences in HPV-positive and negative OPSCC groups [30], [305]. It is possible that although HPV-positive OPSCC is noted in individuals with no history of smoking, they may have a history of tobacco use [39], [54]. However, this group of patients is more likely to have a higher number of sexual partners and oral sex partners [189].

HPV-associated HNSCC arises most commonly in the oropharynx, more specifically, in the lingual and palatine tonsillar crypts unlike those unrelated to HPV which originate from the superficial epithelium [323]. The tonsillar crypt is lined by a specialised lymphoepithelium known as reticulated epithelium. It is hypothesised that this reticulated epithelium provides an immune-privileged site by inhibiting virus-specific T- cells and facilitating immune evasion at the time of initial HPV infection and subsequent virus-induced malignant transformation [323]. Furthermore, disruptions in the reticulated epithelium leave the basement membrane exposed to viral particle deposition without the need for mechanical abrasion of the mucosa [323]. This makes the tonsils more susceptible to HPV infection and subsequent malignant transformation. HPV-associated tumours also have a distinct clinical presentation and differ from non-HPV- related HNSCC in the tumour, nodes, metastases stage distribution at the time of diagnosis (Table 1.6). HPV-positive tumours are generally diagnosed in an earlier T-category and a more advanced N-category than HPV-negative HNSCC [6]. Metastatic lymph nodes from primary HPV- positive tumours in the upper aerodigestive tract often show cystic degeneration at radiological imaging and histological examination. This feature is accepted as a trait of HPV-associated carcinomas, particularly those originating from the base of tongue or tonsil. It is also an indication for diagnostic tonsillectomy and multiple biopsies of base of tongue as part of a clinical work up for occult primary in the head and neck [103]. Sudden changes in the volume of lymph node metastases, with spontaneous shrinkage before and enlargement during radiation treatment, are seen in patients with HPV-related HNSCC [260]. This variability can have implications on clinical staging and assessing treatment complications as a decreasing volume of a target tumour may leave a larger part of normal tissue exposed to radiation. Perhaps the most clinically significant characteristic of HPV-induced tumours in the upper aerodigestive tract is the observation that a certain subset of these tumours particularly those arising in the oropharynx are associated with a better prognosis than those not related to HPV [79].

Histologically too, HPV-positive HNSCC have a distinct profile compared to HPV-negative tumours with the latter tending to be moderately differentiated. HPV-positive tumours are often interpreted as non-keratinising or 'basaloid' carcinomas based on the lobular growth of cells with hyperchromatic nuclei and a high nuclear to cytoplasmic ratio [323]. Table 1.6 is a summary of important differences between HPV-positive and negative HNSCC.

Clinical	HPV-positive HNSCC	HPV-negative HNSCC
Age	Younger individuals	Older individuals
Aetiology	Sexual practices	Tobacco and Alcohol
Anatomic sites	Base of tongue, lingual and palatine tonsils Larynx and other sub-sites (lack of evidence).	All sites
Stage	Tx, T1 – 2	Variable
Survival	Improved	Unchanging
Molecular		
Histology	Non -keratinised	Keratinised
p16	p16 ↑	p16 ↓
p53	p53 wild type	p53 mutated

Table 1.6 Clinical and histopathological differences between HPV-positive and negative HNSCC [322]. Patients with HPV-positive tumours are often younger individuals with no history of smoking or alcohol consumption. Tumours tend to involve the oropharynx and are often non-keratinised with overexpression of p16 and the presence of low levels of wild type p53. Although patients with HPV-positive tumours present with late stage disease, they have improved survival compared to those with HPV-negative tumours.

1.4.3.2 Tumour HPV status and survival

In general, HPV-associated HNSCC at presentation are stage III or IV with multilevel nodal metastases [189], [103]. Despite the late stage presentation, HPV positivity in the oropharynx is associated with improved overall survival and disease free survival [247]. The earliest evidence of a favourable prognosis comes from several small retrospective

case studies which showed that patients with HPV-positive OPSCC, treated by radiotherapy, chemotherapy, surgery or combined therapy had up to an 80% reduction in the risk of disease failure compared to patients with HPV- negative OPSCC [101]. Studies involving retrospective analyses of archival tumour specimens of patients who were enrolled in randomised clinical trials comparing accelerated-fractionation radiotherapy with standard-fractionation radiotherapy, each combined with cisplatin therapy, have also demonstrated a survival advantage in patients with HPV-related OPSCC [5]. In a recent meta-analyses conducted by O'rorke *et al*. the authors observed a 72% reduction in mortality and a 52 – 60% reduction in progression of HPV-positive HNSCC and OPSCC. In addition, HPV-positive HNSCC and OPSCC patients were less likely to have recurrence compared to HPV –negative patients [227]. Better overall survival observed in patients with HPV-positive OPSCC may be attributed to younger age at presentation, lower smoking and alcohol related morbidity, reduced risk of second primary tumours or a more aggressive treatment plan [21]. The presence of a functional wild type p53 and absence of field cancerisation associated with tobacco and alcohol exposure allows an apoptotic response of cancer cells to chemo-radiation [110]. This might explain the favourable treatment outcomes seen in patients with HPV-related OPSCC. Improved treatment outcomes in patients with HPV-associated OPSCC has prompted efforts to identify less aggressive treatment strategies to reduce toxic effects and improve quality of life.

Very little is known about the significance of HPV-mediated carcinogenesis in other head and neck cancer subsites. It is even unclear whether HPV-positive non-oropharyngeal cancers represent another distinct group of viral mediated cancers and whether these patients also have improved clinical outcomes. A recent literature review conducted by Isayeva *et al.* revealed that while high- risk HPV was associated with a significant proportion of oral and laryngeal cancers, there was a paucity of data to implicate the virus in the development of squamous cell carcinomas in other subsites such as the nasopharynx and the salivary glands. Moreover, the existing data is insufficient to establish a correlation between tumour HPV status in non-oropharyngeal sites and clinical outcomes highlighting the need for additional well designed studies to explore the impact of HPV status on overall and disease free survival [127].

1.4.3.3 Tumour HPV status and response to treatment

Several studies show that patients with a positive tumour HPV status are more responsive to treatment than those with negative tumour HPV status. In a study conducted by Kumar *et al.* pre-treatment biopsies of patients with HNSCC enrolled in an organ preserving trial were prospectively analysed for HPV status and EGFR expression. The authors found that patients positive for high-risk HPV had a better response to chemotherapy, better overall survival and disease specific survival [127]. Such preliminary observations were later strengthened by data from prospective clinical trials. In a landmark trial conducted by Fakhry *et al.* in 2008, patients with HPV-positive HNSCC were shown to have higher response rates to induction chemotherapy and chemoradiation treatment with improved overall and progression free survival [79]. Better prognosis seen in HPV-positive HNSCC has been hypothesised to be due to an increased radiosensitivity conferred by low levels of normally functioning p53 in HPV-positive cells following radiation exposure leading to prolonged cell cycle arrest and apoptosis [136] and an impairment of homologous recombination-mediated DNA repair response regulated by p16 overexpression [62]. It has also been postulated that an improved treatment response may be related to an enhanced anti-tumour immune response. For example, Vermeer *et al*. were able to demonstrate that radiation therapy induced a dose-dependent decrease in tumour surface protein CD47 expression. A reduction of CD47 increased phagocytosis of tumour cells by dendritic cells and induced immune activation of mixed lymphocytes [311].

It is important to note that improved outcomes for HPV-related HNSCC were consistently reported for tumours involving the oropharynx where non-surgical treatment modalities are often the norm [159], [253]. Studies analysing the impact of HPV status on prognosis in non-oropharyngeal tumours found no differences in survival or loco-regional control between HPV-positive and negative patients [177], [47], although one must view these findings with caution considering the small sample sizes they were working with.

1.4.3.4 Improving therapeutic strategies for patients with HPV-positive HNSCC

Current treatment protocols for HNSCC are associated with debilitating side effects and long term toxicities. Head and neck cancer survivors have to cope with significant and persistent physical, functional and psychological problems. Laryngeal cancer patients

who undergo chemoradiation or total laryngectomy and radiation therapy will often experience problems with swallowing, chewing, speech and shoulder function [179]. Long-term side effects of xerostomia and problems with dentition have been reported by 5-year survivors of HNSCC [68]. A high level of depression is also common among patients treated for head and neck cancer [207].

Given the younger age and improved treatment performance status of patients with HPV-positive HNSCC, de-intensification of therapeutic strategies is being studied by several groups. Prospective clinical trials focusing on new HPV- specific treatment strategies are underway. Modifying radiation dosage is one way of reducing toxicity and there are currently two ongoing trials, a Phase II trial by Mehrota *et al.* [198] and the Eastern Cooperative Oncology Group Trial (ECOG 1308) [190], focusing on reduction of the standard dose of definitive radiotherapy. In both trials, patients with a positive tumour HPV status received induction chemotherapy followed by a dose reduced chemoradiation for those patients who responded well to induction chemotherapy. Replacement of Cisplatin, currently the drug of choice for chemoradiation, with Cetuximab, a monoclonal antibody against EGFR with less toxicity is an alternative strategy being investigated in the DeESCALaTE HPV and RTOG (Radiation Therapy Oncology Group) 1016 trials [192]. Other novel strategies to reduce morbidity include less invasive surgical procedures like Trans Oral Robotic Surgery (TORS) where surgery is the primary treatment modality and immunotherapy where patients are treated with vaccines intended to stimulate an immune response to the tumour [312], [165].

1.4.3.5 Prophylactic vaccination and patient counselling

The rising incidence of HPV-related HNSCC in many parts of the world has highlighted two important issues; the importance of cancer prevention by way of prophylactic vaccines and the ethics of informed decisions by way of patient counselling.

Currently, the available prophylactic vaccines for oncogenic HPV are indicated for use in women only in the prevention of cervical cancers. The two FDA approved vaccines are Cervarix, a bivalent vaccine manufactured by Glaxo SmithKline and Gardasil, a quadrivalent vaccine manufactured by Merck. While Cervarix is approved for females aged 10 – 12 years and protects primarily against HPV types 16 and 18, Gardasil has been validated for use in females and males aged 9 to 26 years and protects against HPV

types 6 and 11 in addition to types 16 and 18 [211]. As cervical carcinogenesis takes at least 20 years after infection with a high-risk HPV virus, the impact of vaccination programmes on the population would not be expected until 20 to 30 years after implementation. Additionally, screening programmes are able to reduce incidence and mortality of cervical cancers in adult women who are ineligible for vaccination in less developed countries [261].

A higher incidence of oral HPV infection and HPV-positive HNSCC is seen in men [189]. Additionally, HPV infection also increases the risk of anal cancer in men [58]. This has prompted a vigorous campaign for vaccination of boys against HPV. Consequently, the world's first National Human Papilloma Virus (HPV) Immunisation program for boys was launched by the Australian government in 2013 [9]. Presently, a similar vaccination programme for boys is unavailable in the UK. However, such an initiative will come with a caveat that determining its efficacy in the prevention of oropharyngeal cancers will be a difficult, if not impossible, task as these neoplasms are not known to be associated with evaluable pre-cancerous lesions [78].

Presently, determination of tumour HPV status does not impact treatment decisions or patient care other than to provide insight into the disease prognosis. As a result, perhaps, there are no established guidelines for informing patients of the potential link between high-risk HPV infection and cancer. For patients, making informed decisions on treatment choices is dependent partly on receiving accurate medical information. Recommendations have been made to educate patients with HPV-related HNSCC regarding the transmissibility of the virus and the uncertainty concerning health behaviours and epidemiology [279]. Disseminating prognostic information of HPV-related tumours to patients has also been suggested [279]. Attitudes of men toward HPV vaccination need to be clarified in order to draw up education and vaccination campaigns [160].

1.5 Diagnosis of HPV-related HNSCC

It is evident that testing for HPV in HNSCC, especially for those arising in the oropharynx in research settings and in routine clinical practice, is paramount. Indeed, in the light of the knowledge that OPSCC are a distinct subset of HNSCC, the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology (USA) has recommended testing of OPSCC for high-risk oncogenic HPV [222].

Diagnosis of head and neck cancer requires the acquisition of tissue samples or cells in the form of biopsies. Typically, the acquired diagnostic tissue specimens are fixed in formalin, processed in the laboratory and embedded in paraffin wax (Formalin Fixed Paraffin Embedded (FFPE), to preserve morphological features of cells and tissue architecture. This process can sometimes cause degradation of sample DNA, RNA and proteins. HPV tests used in diagnostics must be capable of reliably identifying viral markers in fixed tissue samples in addition to being reproducible and economically viable [256]. In clinical practice, the methods commonly employed for HPV detection are: p16 Immunohistochemistry (IHC), Polymerase Chain Reaction assays (PCR), Quantitative Reverse Transcriptase PCR (qRT-PCR) to quantify viral load and DNA *In Situ* Hybridisation (ISH).

Owing to its simplicity and reliability, p16 IHC is perhaps the most widely used assay to determine the presence of transcriptionally active HPV in OPSCC [166]. The p16 gene, a member of the INK4 class of cell cycle inhibitors, plays an important role as a tumour suppressor whereby it binds to cyclin dependent kinases 4 and 6 and blocks its interaction with D-type cyclins. This allows the tumour suppressor gene retinoblastoma (Rb) to maintain its hypophosphorylated state and bind to E2F transcription factor preventing cell cycle progression [134]. Integration of high-risk HPV into the host genome results in a loss of the viral E2 gene promoter, which in turn causes increased transcription of viral oncoproteins E6 and E7. The E7 protein binds to and inactivates the tumour suppressor Rb protein leading to a compensatory overexpression of nuclear and cytoplasmic p16 in HPV infected tumour cells [290]. PCR based tests rely on the detection of HPV DNA in a given specimen. A DNA polymerase is used to amplify the target gene and the amplified product is visualised on gels or detected by specific probes. Quantitative reverse transcriptase PCR is the current ‘gold standard’ for demonstration of oncogenic HPV infection [283]. By amplifying high-risk E6/E7 mRNA transcripts, it is able to measure viral load in fresh tissue samples. Conventional ISH allows for localisation of expression of a gene in its cellular environment. A labelled DNA probe is used to hybridise to a known target DNA sequence in a given sample. An antibody is then used to detect the label on the probe. As is the case with most testing methods, these diagnostic assays have advantages and disadvantages (Table 1.7).

One of the earliest studies to observe a link between HPV-related squamous cell carcinomas arising in the tonsils and increased levels of p16 was conducted by Klussmann *et al.* in 2003 [139]. The authors determined the HPV status of 34 tumours using consensus and real time PCR and found that a majority of the HPV-positive tumours were strongly positive for p16 by immunohistochemistry while the HPV-negative cases were not [139]. The results of this study have been bolstered by several other studies examining the link between p16 expression and HPV status in HNSCC, making a strong case for using overexpression of p16 in head and neck cancers as a surrogate marker for oncogenic HPV infection [13], [250]. Although p16 IHC is an excellent assay, there are many caveats associated with its use as a single test to determine tumour HPV status. Comparative studies using p16 IHC, ISH and qRT-PCR have demonstrated that although p16 IHC is highly sensitive for HPV infection, it lacks specificity for the presence of transcriptionally active HPV [275], [242]. For example, in a study comparing the prognostic value of qRT-PCR with DNA ISH and p16 IHC in a cohort of OSCC samples, Shi *et al*. reported p16 concordance of 92% and 86% with HPV-16 ISH and qRT-PCR respectively [275]. Some authors argue that in discordant cases, those that are p16-positive but HPV DNA/mRNA negative, p16 overexpression is a result of interplay of other mechanisms unrelated to HPV [264]. Given that overexpression of p16 is a direct consequence of deregulation of Rb, it has been postulated that the loss of heterozygosity of Rb may result in increased p16 expression [258]. The threshold for p16 positivity is currently subjective and disparate. For example, Smeets *et al*. defined p16 positivity as any amount of staining regardless of percentage of tumour cells labelled or staining intensity [283] while Schache *et al.* defined p16 positivity as staining greater than 70% of the tumour [264]. Furthermore, the choice of reagents and the protocol for IHC varies across laboratories and institutions [255]. Additionally, p16 appears to be an effective surrogate marker of high-risk HPV infection only in OPSCC. Its specificity in non-oropharyngeal sites has not been established [175], [46]. Despite the debate on the efficiency of p16 IHC to detect clinically relevant high-risk HPV infection in HNSCC, there is sufficient evidence to support the argument that p16 overexpression is a favourable prognostic indicator of locoregional control and overall survival irrespective of HPV status and treatment modality [5], [253], [306].

PCR based assays are highly sensitive, capable of detecting as little as one copy of a target sequence in a given sample. Therefore, in principle, they are an ideal method for

use on specimens with low DNA content. However, PCR assays are also greatly affected by sample integrity which is especially important in FFPE archival specimens. The presence of foreign material in such a clinical sample can often inhibit the amplification reaction [91]. Because of its high sensitivity, HPV DNA PCR tests tend to overestimate the presence of HPV infection resulting in a few false positive cases. In addition, HPV DNA is widely present in some clinically and histologically normal tissues, head and neck squamous dysplasia, SCCs and even in papillomas [295]. Therefore, detection of HPV DNA in a sample does not make it clinically or biologically relevant. Furthermore, there exists the possibility of false negative results with HPV L1 consensus PCR in cases where L1 ORF (open reading frame) is disrupted as a result of viral integration into the host genome [70]. These observations argue against the use of PCR assays alone in the detection of high-risk HPV infection in HNSCC.

Although qRT-PCR is capable of establishing the presence of transcriptionally active HPV, its utility in clinical practice is limited because of the challenges associated with preservation of RNA in routine biopsy samples [255]. Nevertheless, in a seminal study conducted by Shi *et al*. a qRT-PCR assay developed specifically for FFPE samples was able to detect HPV-16 E6/E7 mRNA transcripts with great accuracy despite the technical challenges of obtaining amplifiable mRNA from the FFPE samples [275].

DNA ISH is specific, permitting direct visualisation of viral DNA in the tumour cells as punctate nuclear signals. Additionally, this assay can be performed on FFPE tissue employing widely available automated IHC platforms and interpreted using conventional light microscopy [16]. However, DNA ISH has limited sensitivity, especially in tumour samples with low viral copy numbers and therefore will fail to detect HPV in them [16]. Hybridisation signals may be faint or scant in some tumour samples making it difficult to interpret the results. Furthermore, the presence of significant background non-specific signal in some DNA ISH platforms can lead to false positives or obscure true positives [16]. There is a general consensus that the detection of transcriptionally active high-risk HPV E6/E7 (i.e detection of E6/E7 mRNA), p16 overexpression and viral integration support a hypothesis of HPV mediated carcinogenesis. In that regard, it must be stressed that DNA ISH merely detects viral DNA and does not provide any information on the presence transcriptionally active high-risk HPV. It has been suggested that p16 IHC may be

employed as a first line assay which when positive can be followed up by either DNA ISH, PCR or both [283], [256], [255].

In addition to the detection methods discussed above, newer assays for the detection of HPV in a diagnostic setting are continually being evaluated. In a breakthrough study, Wang *et al*. described a fluorescent RNA ISH assay, the RNAscope, to detect E6/E7 mRNA of seven high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 52, and 58) using a pool of genotype-specific probes in FFPE tissue samples [315]. The RNAscope has since been validated against the 'gold standard' q-RT PCR by a few groups with excellent analytical and performance in FFPE tissue samples of OPSCC [263], [306],[17].

Method of HPV detection	Advantages	Disadvantages
p16 IHC	1. Easy to perform in labs. 2. Ease of interpretation of results. 3. Highly sensitive 4. Relatively inexpensive	1. Low specificity 2. Low validity in detecting HPV infection in non-oropharyngeal sites
PCR	1. Highly sensitive and specific 2. Consensus primers can detect a wide spectrum of low-risk and high-risk HPV types	1. High sensitivity can lead to false negatives 2. Cannot distinguish between transcriptionally active and inactive HPV 3. Primers detect sequences in the L1 region which may be lost upon integration of the virus into the host genome. Therefore, PCRs may give false negative results if the virus is in the integrated form
qRT-PCR	1. Highly sensitive and specific	1. Requires intact RNA 2. Requires considerable technical expertise 3. Optimally performed on fresh frozen samples

***In Situ* Hybridization**	1. Highly specific 2. Compatible with standard automated IHC platforms and FFPE sections 3. Results can be interpreted in context to the tissue 4. Results can be visualized on a standard microscope	1. Low sensitivity 2. Interpretation of results is sometimes difficult

Table 1.7 Pros and cons of current HPV testing methods [16].

1.5.1 Standardising HPV testing practices

Establishing high-risk HPV infection in HNSCC patients is crucial to determining its true prevalence, risk stratification, predicting clinical outcomes, patient counselling and understanding more about the natural history and molecular underpinnings of the disease. However, HPV testing in routine practice and research settings is not directed by standardised guidelines and protocols. Working groups such as the American Joint Committee on Cancer (AJCC) and the Royal College of Pathologists, UK now recommend reporting HPV status for OPSCC in routine clinical practice [255]. Clinical guidelines for treatment of head and neck cancers only necessitates determination of HPV status for OPSCC as there is insufficient evidence to recommend testing at other subsites such as the oral cavity and the larynx [255]. In order to assign a diagnosis of HPV positivity, the clinical specimen must harbour high-risk HPV DNA along with evidence of viral oncogene expression. Assays currently employed for diagnosis such as PCR, DNA ISH, and p16 IHC vary considerably in detection rates making it difficult to compare the results of existing prevalence studies [28]. Furthermore, some of these tests are economically non-viable, riddled with technical challenges and are not governed by standardised guidelines for interpretation of results as seen in the case of p16 IHC. They are, therefore, selected based on the resources and facilities available to each laboratory [255]. In 2013, we conducted an online survey to gather information about practice trends related to HPV testing for HNSCC across the Scottish NHS centres (Unpublished data, see Appendix A). We found that most NHS centres were employing p16 IHC alone or in combination with PCR for routine detection of high-risk HPV in HNSCC. In addition, we noted that p16 IHC reporting criteria was inconsistent and disparate across the NHS boards. There is a general consensus that the aforementioned diagnostic assays cannot be used alone and must be used in combination for optimal HPV status determination. Where only FFPE samples are available, a combination of p16 IHC and PCR/DNA ISH is recommended [256]. While such a diagnostic algorithm can be easily

adopted by most pathology laboratories, the techniques, their protocols and reporting criteria must be standardised and bound by stringent quality assurance measures [256].

1.5.2 Identification of novel markers of high-risk HPV-related HNSCC

In cancer research and diagnostics, biomarkers are invaluable tools to identify the presence of a tumour, to determine patient prognosis and for treatment selection. Personalised medicine employing predictive markers could improve outcomes and encourage better treatment planning. In HNSCC, a definitive example of disease management with the help of biological markers is the targeting of EGFR with the monoclonal antibody, Cetuximab [20]. Extensive studies on cervical carcinogenesis have led to the identification of certain host molecules, involved in cell cycle regulation, which are altered by the HPV oncoproteins [244], [287]. These candidate biomarkers have shown promise in improving diagnostic accuracy for cervical cancer. Unfortunately, biomarker studies on HNSCC related to high-risk HPV are few and far in between.

Detection of viral E6/E7 oncogene transcripts is regarded as the gold standard for the presence of clinically relevant high-risk HPV infection. Detection of E6/E7 mRNA has, however, proven to be very challenging using the conventional methods [17]. In order to establish a causal link between the virus and HNSCC, pathologists must be able to review the specimen and assess the marker within the tissue microenvironment of clinical specimens. Recent advances in HPV biomarker detection like the visualisation of E6/E7 mRNA using RNA ISH (RNAscope) have shown promise in providing a highly sensitive and specific platform for detection of HPV biomarkers in HNSCC samples [263], [28].

Investigators have also sought to identify alternate biomarkers for clinical assessment of HPV-related HNSCC. For instance, in a study conducted by Liang *et al*. in 2012, the authors attempted to establish a correlation between serological markers of HPV infection and HNSCC. The presence of antibodies to HPV-16 L1, E6 and E7 was determined for 488 patients with HNSCC. Immunohistochemical analysis of p16 expression was determined in a subset of 233 cases and the presence of HPV-16 DNA was determined by PCR in a subset of 179 cases. Each biomarker was considered individually in the subset of patients studied for all endpoints. The authors found that seropositivity for E6 and E7 proteins was significantly associated with enhanced overall

survival in oropharyngeal cancer both as a single assay and in combination with HPV DNA PCR [170]. In another example, Mroz *et al.* examined next generation sequencing data and associated clinical records of a cohort of 302 HNSCC patients with 32 HPV-positive cases. The researchers found that mutant-allele tumour heterogeneity (MATH) scores for HPV-positive tumours were lower compared to HPV-negative HNSCC patients [208]. Intra-tumour heterogeneity or variation between tumour cells within a patient's tumour is often a source of treatment resistance and failure. MATH is a marker of intra-tumour heterogeneity based on tumour next generation exome sequencing and is an indicator of poor outcome [257]. Thus, MATH scores in HPV-positive tumours indicate a more homogeneous tumour and improved clinical outcomes.

1.6 Molecular targets of high-risk HPV and their validity as biomarkers

The E6 and E7 high-risk viral oncoproteins promote cellular transformation by interacting with and inactivating several cellular proteins, most importantly the tumour suppressor proteins p53 and pRB [64]. E6 is a small protein, with a molecular weight of 18 kDa, composed of two zinc binding domains and a highly conserved C-terminal PDZ binding motif through which it interacts with its cellular binding partners [204]. On the other hand, E7 is a nuclear protein of 98 amino acids divided into three domains or conserved regions (CR), CR1, CR2 and CR3. CR1 is composed of amino acids 1–20, CR2 contains residues 21–39, and CR3 has 40–98 amino acid residues. E7 dimerizes in the CR3 domain via a zinc-finger motif which is essential for proper functioning of the protein [41].

The ability of high-risk E7 to bind to members of the retinoblastoma protein (pRb) family, which play an important role in cell cycle regulation, is perhaps the most crucial property of this viral oncoprotein. Hypophosphorylated pRb binds to transcription factors of the E2F family and represses the transcription of certain cell cycle genes. When cells progress from G0 through G1 to S-phase, pRb gets hyperphosphorylated by G1 cyclin–dependent kinases, releasing the transcription factor E2F, which in turn activates genes involved in DNA synthesis and cell cycle progression [72]. E7 binds to hypophosphorylated pRb and thereby induces cells to enter into premature S-phase by disrupting Rb-E2F complexes [123], [238], [327]. E7 also contributes to growth stimulation of infected cells by interfering with the inhibitory activities of cyclin kinase

inhibitors p21 and p27 [330]. E7 also associates with and influences other cellular proteins (Table 1.8).

Binding partner	Consequence of interaction
Activator protein (AP1) family members	Abrogation of interferon regulatory factor-1 (IRF-1) transcriptional activity
c-myc	Enhancement of c-myc-induced transcription
Insulin growth factor binding protein-3 (IGFBP3)	Anti-apoptotic effect Stimulation of cell proliferation
p21	Growth stimulation via deregulation of cell cycle
p27	Growth stimulation via deregulation of cell cycle
Subunit4 ATPase	pRb degradation via proteasome

Table 1.8 Molecular interactions of HPV oncoprotein E7 [23]. Besides its interaction with the pRB protein, the E7 oncoprotein of high-risk HPV types target various other cellular proteins and disrupt multiple cellular pathways.

The primary target of the E6 oncoprotein, p53, is a transcription factor which stimulates the expression of genes such as the cyclin dependent kinase inhibitor (CDK1) which is involved in cell cycle arrest and apoptosis. The E6–p53 interaction is mediated by E6AP (E6 associated protein), an E3 ubiquitin ligase which facilitates binding of E6 to p53 leading to ubiquitin mediated degradation of the latter [265]. E6 binding of p53 also inhibits its translocation into the nucleus preventing it from activating or repressing target genes [201].

The E6 protein also contributes to carcinogenesis by disrupting multiple cellular pathways, one of which is mediated through its interaction with PDZ (Postsynaptic density 95, PSD-85; Discs large, Dlg; Zonula Occludens-1, ZO-1) domain proteins [191]. A study conducted by Simonson *et al.* in an E6 transgenic mouse model showed that interaction of E6 with its PDZ domain partners induced spontaneous tumours and contributed to the promotion stage of skin carcinogenesis, suggesting that the ability of E6 to target PDZ proteins is an important step in tumourigenesis [281]. HPV E6 proteins interact with a large number of PDZ domain containing cell proteins through their C- terminal PDZ binding motif. Whether this interaction is always mediated by E6AP is unclear. For example, Grm and Banks [109] reported that E6 degradation of

Dlg and MAG1 family of proteins is E6AP independent in vitro, while a study conducted by Kuballa *et al*. [151] in 2007 showed that E6 mediated degradation of Dlg depends on the presence of E6AP within cells. Apart from the interactions discussed above, the E6 oncoproteins also target other molecules to disrupt the normal functioning of cells (Table 1.9).

Binding partner	Consequence of interaction
Pro-apoptotic protein Bak	Anti-apoptotic effect
Pro-apoptotic protein Bax	Anti-apoptotic effect
CREB binding protein (CBP/p300)	Downregulation of p53-dependent transcription.
c-myc	Prevention of myc-induced apoptosis. Increases hTert transcription and telomerase activity.
hDlg	Deregulation of cell cycle. Loss of cell differentiation.
hScrib	Influence on cell adhesion and polarity
Paxillin	Disruption of actin cytoskeleton and cell matrix Interactions

Table 1.9 Molecular interactions of HPV oncoprotein E6 [23]. The E6 oncoprotein of high-risk HPV types promotes carcinogenesis by targeting various cellular proteins and disrupting multiple cellular pathways.

It is evident that malignant transformation of epithelial tissues by high-risk HPV involves multiple cellular pathways, all of which require overexpression of E6 and E7. Naturally then, E6 and E7 proteins have been the main focus of HPV screening, diagnostic and prevention methods. Diagnostic methods such as PCR and qRT-PCR rely on the detection of the E6/E7 genes and mRNAs respectively. E6 and E7 proteins are also primary targets in the development of therapeutic HPV vaccines and gene therapy [184], [277].

1.6.1 Impact of high-risk HPV E6 on PDZ domain proteins

Epithelial cells have a characteristic polarised architecture and are bound by specialised cell-cell junctions, including desmosomes, tight and adherens junctions [317]. This cell

polarity plays an important role in the organisation of signal transduction pathways that control important cellular processes such as proliferation, motility, differentiation and apoptosis and is maintained by protein complexes namely the Crumbs, Par and Scribble complexes [245], [156]. The Crumbs complex, made up of Crumbs, Pals1 (protein associated with Lin seven 1) and PatJ (PALS1-associated tight junction protein) maintains apico-basal polarity [245]. The Par complex which consists of Par3, Cdc42, Par6 and atypical protein kinase C (aPKC) is a dynamic complex and also interacts with the Crumbs complex. Scaffolding proteins Scribble (Scribbled planar cell polarity protein or Scrib), Dlg and Hugl form the Scribble complex which maintains basolateral polarity [245]. Loss of cell polarity, either through degradation or mislocalisation of the component proteins, is a key step in the transition of a benign tumour to a malignant one. It may lead to mislocalisation of receptors causing aberrant signalling or a redistribution of cell adhesion molecules promoting Epithelial-Mesenchymal-Transition (EMT). It may also lead to altered distribution of matrix metalloproteinases at cell surfaces promoting cell invasion and migration [104].

Cell polarity is dependent on the correct spatio-temporal regulation of the expression levels of the cell polarity regulators many of which are PDZ domain containing proteins [89]. The PDZ domains, consisting of approximately 80-90 amino acid residues, serve as scaffolds for protein assembly and interactions. They have a well-defined interaction "pocket" which can be filled by a PDZ-binding motif (PBM) "ligand". The PDZ-binding motifs are specific sequences often located at the carboxyl terminus of certain proteins and facilitate interactions with PDZ domains [213]. Therefore, PDZ domain proteins are vital to cell polarity and the formation of adherens and tight junctions, molecular scaffolding for assembly of protein complexes and tumour suppressor activity [53], [80]. For instance, Dlg1 and PATJ, both PDZ domain proteins, are important in the formation of anterior posterior polarity in T cells and epithelial cells [278], [150]. The role of Dlg1 in limiting cell proliferation in Drosophila is indicative of a parallel function as tumour suppressor in mammalian cells. The loss of the Scribbled planar cell polarity protein (Scrib), a similar cell polarity protein, has been associated with tumour progression and invasiveness [125].

PDZ domain containing proteins are common targets for inactivation by oncogenic viruses [24]. A unique feature of the E6 proteins of high-risk HPVs is the presence of a highly conserved class I –PBM located at the C-terminus. In contrast, PBMs are not

present in the E6 proteins of low-risk HPV types indicating that the PBM is a molecular signature for the malignant potential of high-risk HPV types [137], [82]. Furthermore, PBMs also appear to play an important role in the viral life cycle especially in the maintenance of viral copy numbers and of viral DNA as episomes in undifferentiated cells [162]. Through its C-terminal PBM, the E6 oncoprotein can interact with several PDZ domain proteins. Such interactions often interfere with the establishment of epithelial cell polarity and lead to loss of epithelial cell organisation thus contributing to malignancy [10]. One of the first PDZ targets of high-risk HPV E6 to be reported is hDlg1. It was found that degradation of hDlg1 by E6 oncoprotein contributed to epithelial cell proliferation and dysplasia in cervical carcinogenesis [10]. Apart from Dlg, several other PDZ domain substrates of high-risk HPV E6 have been described and these interactions affect diverse consequences ranging from loss of cell polarity and cell–cell attachment, and de-regulation of various cell signalling pathways (Table 1.10).

Protein	Function	Effect on the target protein
Membrane Associated Guanylate Kinase Inverted (MAGI) group of proteins	Cell Polarity Tumour Suppressor PTEN localisation to membrane	Ubiqutination and proteasomal degradation
Multiple PDZ domain protein-1 (MUPP-1)	Scaffold Protein	Ubiqutination and proteasomal degradation
PATJ	Tight junction formation integrity	Ubiqutination and proteasomal degradation
PSD95	Signalling complex scaffold at synaptic junction	Ubiqutination and proteasomal degradation
NHERF1/EBP50	PI3K/AKT signalling	Ubiqutination and proteasomal degradation
TIP-2/GIPC (Tax Interacting Protein, clone 2/GAIP Interacting Protein, C terminus)	TGFβ signalling	Ubiqutination and proteasomal degradation
Cystic fibrosis transmembrane conductance regulator-associated ligand (CAL)	Intracellular trafficking	Transcriptional repression

Table 1.10 Functions and outcomes of PDZ domain substrates of the high-risk HPV E6 oncoprotein [302]. The E6 oncoprotein of high-risk HPV types interact with and interfere with the function of several PDZ domain proteins to affect diverse consequences ranging from loss of cell polarity and cell-cell attachments to deregulation of various cell signalling pathways.

Interestingly, the affinity with which high-risk E6 proteins target their substrates is dependent on their PBM and minor alterations therein. For instance, hDlg is

preferentially targeted by HPV-18 E6 while hScrib is targeted by HPV-16 E6 [302]. Furthermore, E6 preferentially targets specific cellular pools of PDZ domain containing proteins for proteasome degradation [191].

1.7 Ezrin-Radixin-Moesin Binding Protein 50

The focus of this thesis is a PDZ domain containing substrate of the high-risk HPV E6 protein called Ezrin-Radixin-Moesin Binding Protein 50 (EBP50). EBP50 was first identified as a cofactor for inhibition of the Na+/H+ exchanger 3 by PKA (Protein Kinase A) in the renal brush border membrane and was also found to associate with high affinity to members of the ERM protein family (Ezrin-Radixin-Moesin) [320], [249] The ERM proteins play a crucial role in the organisation of complex membrane domains by interacting with transmembrane proteins and the underlying cytoskeleton [81]. EBP50 stabilises the ERM proteins at the plasma membrane, serving to facilitate their role in strengthening the cell cortex and regulating signal transduction pathways. It also functions as a molecular scaffold, promoting the assembly of macromolecular protein complexes at the apical membrane of epithelial cells. For example, the stabilisation of membrane receptors like PDGFR (Platelet Derived Growth Factor) and G protein coupled receptors and the turnover of ion transport proteins like CFTR (Cystic Fibrosis Transmembrane Regulator) is mediated via their interaction with EBP50 [95].

The structure of EBP50 comprises of tandem and homologous PDZ domains in which amino acids 11-97 constitute PDZI and amino acids 150-237 constitute PDZ II. The C-terminal ERM binding region (EB) is made up of 30 amino acids. EBP50 interacts with more than 30 proteins, some of which have been implicated in human diseases such as cystic fibrosis, chronic kidney disease and cancer [95], [94]. Most protein-protein interactions takes place through PDZ I (Figure 1.4). However, some proteins bind specifically to PDZ II [206]. EBP50 normally adopts an intramolecular, folded conformation in which the C-terminal EB region binds to the PDZ II masking the interaction of this domain with its binding partners [206]. This head-to-tail folding is facilitated by the presence of a consensus PDZ motif in the EB region of EBP50 (Figure 1.4). The ERM proteins bind to the EB region of the EBP50 disrupting the folded conformation and thereby allowing the association of proteins to the PDZ domains to form macromolecular protein complexes [15]. EBP50 interactions with its protein partners are regulated by phosphorylation. For example, Serine 289 (Ser 289) is

phosphorylated by GRK6A (G protein coupled receptor kinase A) which binds to PDZ I of EBP50. Additionally, EBP50 is phosphorylated at Serine 279 (Ser279) and Serine 301(Ser301) by cyclin dependent kinase, Cdc2 [115].

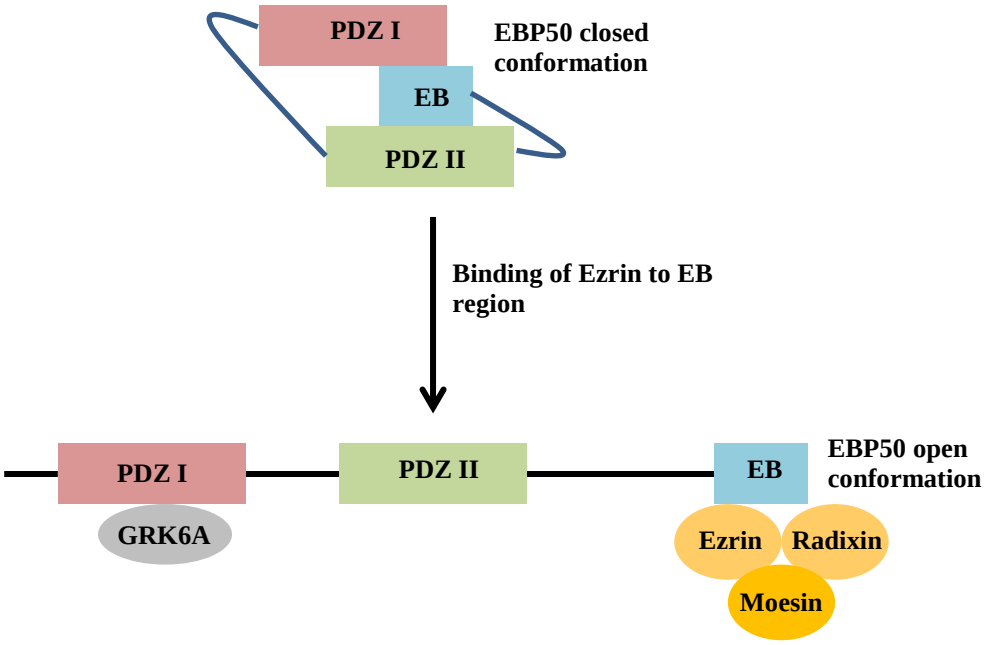

Figure 1.4 Structure of EBP50 (inactive and active conformations). EBP50 comprises of tandem and homologous PDZ domains, PDZ1 and PDZII, and a C-terminal ERM binding region (EB). Most protein interactions take place through PDZ1 (GRK6A is shown binding to PDZ1). EBP50 normally adopts an intramolecular, folded conformation in which the C-terminal EB region binds to the PDZ II masking the interaction of this domain with its binding partners. Unfolding of this closed conformation is facilitated by binding of ERM proteins to the EB region of the EBP50 and thereby allowing the association of proteins to the PDZ domains to form protein complexes.

1.7.1 The role of EBP50 in cancer

It has been widely speculated that EBP50 is an important player in cancer. However, its exact role in tumourigenesis is controversial. Under physiological conditions EBP50 localises to the apical membrane of polarised epithelial cells where it stabilises transmembrane receptors and junctional complexes through interactions with its PDZ domains and with ERM proteins through the EB region [205]. Aberrant EBP50 expression has been observed in numerous malignant tumours including breast cancer, colorectal cancer, and hepatocellular carcinoma with EBP50 reported to be either absent or overexpressed in an altered intracellular distribution [288], [114], [276]. Such expression patterns led to a postulation that EBP50 may act as a tumour suppressor when it is localized at the plasma membrane and as an oncogenic protein when it is localized in the cytoplasm [95]. A study conducted by Molina *et al.* revealed that

EBP50 was mislocalised from the plasma membrane to the cytoplasm in Glioblastoma Multiforme resulting in destabilisation and inactivation of PTEN (Phosphatase and tensin homologue), a key suppressor of the PI3 kinase/ Akt pathway [203]. In another study, conducted on mouse embryonic fibroblasts, Kriemann *et al*. show that cortical stabilisation of β-catenin by EBP50 is essential to suppress anchorage independent growth [146]. These studies support a tumour suppressor role of EBP50. In contrast, Shibata *et al*. noted overexpression of EBP50 in hepatocellular carcinomas. Furthermore, they observed that EBP50 overexpression promoted Wnt signalling by enhancing β-catenin/TCF mediated transcriptional activation [276]. An overexpression of EBP50 with relocalisation to the cytoplasm was observed in Estrogen Receptor (ER) positive breast cancers where it was associated with metastatic progression and poor prognosis [94]. The EBP50 gene promoter contains Estrogen Response Elements (ERE) which might explain increased expression in estrogen receptor positive breast tumours [73]. These studies indicate that overexpression of EBP50 may have an oncogenic role and may also be associated with tumour stage, metastasis and poor prognosis. The contrasting reports from these studies indicate a possible dual role of EBP50 as an oncogene and tumour suppressor depending on its localisation at the plasma membrane or its redistribution to the cytoplasm. Indeed, Georgescu *et al*. have proposed a reconciliatory model to illustrate the involvement of EBP50 in cancer. According to the authors, EBP50 serves as a tumour suppressor in normal cells by recruiting and stabilising membrane protein complexes including PTEN tumour suppressor and β-catenin thereby restricting signalling downstream of growth factor receptors and merging β-catenin/E-cadherin complexes at the plasma membrane. By recruiting PTEN to the membrane, EBP50 facilitates the formation of a complex by bridging PTEN to PDGFR. This restricts the activation of the PI3K (Phosphoinositide 3 kinase) pathway following activation by PDGF (platelet derived growth factor [94]. Disruption or absence of EBP50 at the plasma membrane could interfere with its growth suppressive effects as evidenced by prolonged activation of the PI3K pathway and increased migration of MEF cells (mouse embryonic fibroblasts) in response to PDGF [296]. Although, the oncogenic effect of EBP50 when it is overexpressed in the cytoplasm of tumours requires further elucidation, it is likely to stem from a failure to consolidate protein complexes at the plasma membrane or an organisation of complexes with different ligands in the cytoplasm [95].

1.7.2 Mechanism of EBP50 regulation by high-risk HPV

The discovery of EBP50 and its role in carcinogenesis, coupled with the knowledge that PDZ domain containing proteins are targeted by high-risk HPV E6 proteins, led to speculations that EBP50 might in fact be a substrate of high- risk HPV E6 oncoprotein. Accardi and colleagues were the first to investigate this possibility and the authors found that HPV-16 E6 associated with EBP50 via the C- terminal PBM and promoted its degradation *in vitro* [2]. Surprisingly, a similar interaction was absent between HPV-18 E6 and EBP50, despite possessing nearly identical PBMs, indicating that EBP50 degradation was exclusive to the E6 oncoprotein of HPV-16 and also reiterating previous reports that E6 proteins do not target their PDZ domain containing substrates with equal affinity [2], [302]. The authors also noted that degradation of EBP50 was assisted by the E7 oncoprotein which by activation of the cyclin-dependent kinase complexes, promoted the accumulation of a phosphorylated form of EBP50, which was preferentially targeted by E6. As with other PDZ targets of the E6 oncoprotein, degradation of EBP50 was also mediated by the E6AP ubiquitin ligase *via* the proteasome pathway [2]. Results of *in vivo* studies showed that EBP50 protein levels were downregulated in HPV-16 positive cervical cancer derived cell lines; SiHa and CaSki as well as in cervical intraepithelial neoplasia (CIN) corroborated the *in vitro* findings. Furthermore, HPV16 E6 mediated degradation of EBP50 correlated with the activation of the phosphatidylinositol-3′-OH kinase (PI3K)/AKT signalling pathway[2].

Data from our lab has also identified EBP50 as a novel target for high-risk HPV-16 E6 oncoprotein [237] and our findings are consistent with the report published by Accardi *et al*. Results of *in vitro* studies on H1299 cells (human non-small cell lung carcinoma cell line) transfected with high-risk HPV-16 E6 expression plasmids showed decreased EBP50 levels. On the contrary, the levels of EBP50 protein remained relatively unchanged following transfection of H1299 cells with control plasmids pcDNA3 and pcDNA3 Hdm2 suggesting that degradation of EBP50 was brought about by high-risk HPV-16 E6.

It is evident that HPV-16 interferes with EBP50 function by targeting it for proteasome mediated degradation. Data from the report published by Accardi *et al*. also indicate that EBP50 expression may be an early marker of cervical carcinogenesis [2]. Whether such a role exists for EBP50 in the development of HNSCC, a small proportion of which are associated with high-risk HPV, is a question that merits investigation.

1.7.3 Impact of EBP50 deregulation by high-risk HPV

Given that there is currently very little information available on the biological impact of EBP50 regulation by the high-risk HPV E6 protein, we can only speculate based on extrapolation of results from studies on other well characterised E6 substrate PDZ domain proteins such as the MAGI family of proteins, hDlg1 and hScrib.

High-risk E6 protein interaction with MAGI-1, a key component of epithelial tight junctions[212], offers a more probable conjecture for HPV- induced carcinogenesis in the context of PDZ domain protein targets. Findings of a systematic analysis by Kranjec and Banks indicate that the E6 proteins of HPV-16 and 18 target membrane bound forms of MAGI-1 resulting in the loss of epithelial tight junctions [145]. Using ZO-1 (zonula occludens) a marker of tight junction integrity, they demonstrated that these junctions are mostly absent in HPV-18-positive HeLa cells and that ablation of E6 expression results in a re-accumulation of MAGI-1 at the cell membrane and restoration of tight junctions [145]. Tight junctions play an important role in keratinocyte differentiation by promoting exit from the cell cycle [3]. Additionally, they participate directly in the regulation of cell proliferation by modulating signalling cascades such as mitogen-activated protein kinase (MAPK), PKB/Akt, and RhoA signalling [168], [142], [3]. Thus, binding to MAGI-1 through its PBM motif, may allow the virus to stabilise E6 protein expression in the early stages of its life cycle [226], [213]. Additionally, targeting MAGI-1 for proteasome mediated degradation allows the virus to undermine tight junctions and promote cancer progression by inducing EMT like morphological changes in the epithelial cells [213].

PDZ domain containing proteins hDlg and hScrib are key components of the epithelial adherens junctions and play an important role as tumour suppressors [213]. By binding to hDlg and hScrib in the early stages of the viral life cycle through its E6 PBM, HPV-16 has been shown to be able to persist in its episomal state [226]. The E6 oncoprotein appears to differentially regulate the localisation and expression of both hDlg and hScrib as evidenced in the progression of cervical cancer from cervical intraepithelial lesions to invasive cancer. A redistribution of these proteins from sites of cell-cell contact to the cytoplasm is observed in precursor lesions followed by a gradual reduction in expression and complete loss in invasive cancer [215]. Interestingly, it appears that E6 preferentially targets a specific sub-cellular pool of hDlg. In a study conducted by Massimi *et al*., the authors found that E6 specifically targeted nuclear

pools of the protein rather than those found within multiprotein complexes at cell-cell junctions [191]. It has been postulated that certain post-translational modifications of these PDZ domain proteins, more specifically phosphorylation, affect their localisation and make them more susceptible to degradation by the E6 oncoprotein. For instance, when phosphorylated by CDK1 and CDK2 at Ser158 and Ser442, hDlg accumulates in the nucleus making it more susceptible to the E6 protein [216]. The biological consequences of sub-cellular location specific PDZ protein-E6 interactions are unclear. Thus, interaction of high-risk HPV E6 with PDZ domain proteins allows the virus to sustain a chronic persistent infection in the host cell, abrogates the tumour suppressive function of these proteins, induces loss of cell-cell contact by undermining the integrity of tight and adherens junctions and promotes cell proliferation and migration.

EBP50 functions as a scaffolding molecule to stabilise β-catenin-E-cadherin complexes at the plasma membrane and contributes to the integrity of adherens junctions [146]. It also links tumour suppressor PTEN with PDGFR in a ternary complex which attenuates the activity of the PI3K/Akt pathway [95]. In the absence of evidence, a conjectural biological consequence of EBP50 degradation by the E6 protein is disruption of β-catenin-E-cadherin complexes and the subsequent disintegration of adherens junctions in epithelial cells. It may also facilitate epithelial mesenchymal transition and increased cell migration and invasiveness [124]. Regulation or loss of EBP50 by E6 may also result in the prolonged activation of the PI3K/Akt pathway in response to PDGF stimulation.

1.7.4 Current role of EBP50 as a biomarker

As described in section 1.7.1, EBP50 appears to have a dual function depending on its subcellular distribution - that of a tumour suppressor protein when it is localised at the plasma membrane or an oncogene when it is shifted to the cytoplasm. Expression patterns and distribution of EBP50 have been studied by several authors in various human tumours employing different methodologies such as immunohistochemistry, ISH and immunofluorescence [285]. These studies show differential expression patterns and suggest an emerging role for EBP50 as a potential tumour marker.

The earliest evidence with respect to EBP50 as a potential biomarker was provided by Shibata and colleagues. The authors found that EBP50 associated with β-catenin to promote Wnt signalling in hepatocellular carcinoma cell lines. The authors also noted

overexpression of EBP50 in surgical specimens of hepatocellular carcinoma suggesting that EBP50 may work with β - catenin to drive tumour formation in liver cancer [276]. Indeed, aberrant activation of Wnt/β-catenin signalling has been reported in a wide range of hepatocellular carcinomas [220].

EBP50 expression has been studied extensively in breast cancer. A major cause of death in women, breast cancer has varied clinical outcomes and response to treatment. Loss of heterozygosity at the EBP50 gene locus is seen in more than 50% of the tumours and very rarely in other types of cancer. Although, differential EBP50 expression has been described in other cancers such as hepatocellular carcinoma and schwannoma, the data is most representative for breast cancer [14]. For instance, Cardone *et al*. analysed EBP50 expression in human breast tumours and contiguous normal tissues from the same patients and found that EBP50 was overexpressed in all the tumours when compared to the adjacent normal lobules [35]. In yet another study, Mangia *et al*. described a change in subcellular localisation of EBP50 wherein, EBP50 was mostly localised in the cytoplasm of epithelial cells in primary or metastatic tumours compared to membranous immunoreactivity seen in the luminal aspects of normal breast epithelial cells. Additionally, they also noted that cytoplasmic/membranous EBP50 expression ratio progressively increased from ductal carcinoma *in situ* to primary invasive cancer suggesting that it plays a role in breast cancer progression [187], [288]. It was also noted that EBP50 expression significantly increased with increasing tumour histological grade and indicated poorer prognosis in breast cancer [285].

In addition to hepatocellular carcinoma and breast cancer, the prognostic impact of EBP50 expression has been evaluated in other tumours such as gastric, pancreatic, colorectal, prostate cancer and glioblastomas (Table 1.11).

Type of cancer	EBP50 expression pattern	Clinical significance	Reference
Hepatocellular carcinoma	↑↑	Indicates a tumour suppressor function by inhibiting anchorage independent growth in hepatocellular carcinoma cell lines	Xiu-lan peng *et al.[241]*
Breast cancer	↑↑	Increasing tumour histological grade, aggressive clinical behaviour, unfavourable prognosis, and increased tumour hypoxia	Bellizi *et al.*[14]
Gastric cancer	↑↑	Correlates with tumour size	Xiao Guang *et al.* [183]
Pancreatic cancer	↑↑ Cytoplasmic overexpression	Indicates a tumour suppressor function by inhibiting uncontrolled cell growth and proliferation	Ji Meng *et al.* [130]
Colorectal cancer	↑↑ Cytoplasmic overexpression	Loss of membranous staining from colorectal adenoma to colorectal cancer and cytoplasmic overexpression in colorectal cancer	Hayashi *et al.[114]*
Glioblastoma	↑↑ Cytoplasmic overexpression	Loss of membranous staining from normal astrocytes to glioblastoma multiforme with cytoplasmic overexpression in the latter	Molina *et al.[203]*
Prostatic adenocarcinoma	↓↓	Progressive decrease in membranous staining from benign disease to metastatic pancreatic cancer. Indicative of tumour suppressor function?	Barthlow *et al.[11]*

Table 1.11 EBP50 expression and its clinical significance in human cancers. The prognostic significance of EBP50 expression has been studied extensively in breast cancer. It has also been evaluated in hepatocellular carcinomas, gastric, pancreatic and colorectal cancers, glioblastomas and prostatic adenocarcinomas.

1.7.5 Study Objective: Investigating the validity of EBP50 as a potential marker for HPV-related HNSCC

HPV-mediated HNSCC has emerged as a significant health threat and poses unique epidemiological and biological challenges. Tumour HPV status has important implications in the determination of true disease prevalence, disease prognosis, its natural history and molecular pathogenesis. While HPV positivity is more commonly detected in OPSCC where it confers a survival advantage and improved treatment response in patients [84], its prevalence and clinical impact in other head and neck subsites remains largely unexplored. Current head and neck cancer treatment regimens carry a significant risk of debilitating side-effects and morbidity prompting researchers to investigate less intensive therapeutic strategies for patients with HPV-related HNSCC. However, HPV risk stratification is impaired by a lack of standardised guidelines and robust testing methods for diagnosis and the unavailability of additional molecular markers. Furthermore, there is a dearth in our understanding of the molecular mechanisms underlying HPV driven tumour development in both oropharyngeal and non-oropharyngeal sites.

As discussed earlier the E6 and E7 proteins of high-risk HPV promote carcinogenesis by disrupting multiple cellular processes, the most important cellular targets being the p53 and pRB proteins. Apart from these interactions, the HPV E6 oncoprotein also targets PDZ domain proteins which act as adaptors and stabilise protein complexes involved in cell signalling, cell-cell adhesion and cell polarity at the plasma membranes of cells [302]. E6 mediated degradation of PDZ domain proteins has been shown to result in aberrant cell signalling and disruption of epithelial cell-cell attachments possibly triggering invasion and metastasis [10] , [145]. Data from our lab indicates that the PDZ domain protein EBP50 is targeted for degradation by the high-risk HPV E6 protein resulting in a downregulation of the protein levels in cells [237]. EBP50 has already emerged as a molecular marker in the development of breast cancer [94], colorectal cancer [114] and hepatocellular carcinoma [276] however, the possibility of this scaffolding protein as a potential marker in HPV-related HNSCC has not been explored previously. Therefore, it was natural to base this thesis on the following hypothesis:

The downregulation of EBP50 may contribute to HPV-mediated malignant transformation of epithelial tissues in the head and neck region making EBP50 a potential marker for HPV-related HNSCC.

In particular, this study proposes to test whether EBP50 distribution is influenced by tumour HPV status in a sample population of HNSCC patients in Tayside, Scotland.

The main objectives and specific aims of this study are:

1. Determination of the incidence of high-risk HPV and its impact on clinical outcomes in a sample population of HNSCC patients in Tayside, Scotland. The study achieves this by -

   A) determining tumour HPV status of a selected cohort of HNSCC patients

   B) determining the concordance between high-risk HPV DNA positivity and p16 status in the cohort

   C) linking tumour HPV status to clinical outcomes such as survival and recurrence in the cohort.

2. Evaluation of the validity of EBP50 as a novel marker for HPV-related HNSCC. This is done by -

   A) analysing differences in expression patterns of EBP50 in high-risk HPV-negative and positive patient samples

   B) determining if there is a correlation between EBP50 expression patterns and clinical outcomes such as survival and recurrence.

Chapter 2

RESEARCH METHODOLOGY

2.1 Cell Culture

2.1.1 Materials

The 3T3 cell line, a fibroblast cell line derived from Swiss mouse embryonic tissue was a kind gift from Dr. Dorothy (Sam) Crouch (Unit of Cell and Molecular Biology, Dundee Dental School). The HaCaT cell line, derived from human keratinocytes was a kind gift from Professor Irwin McLean (Division of Molecular Medicine, University of Dundee) and was generated as described in Boukamp *et al* [22]. The SiHa cell line expressing Human Papillomavirus type 16 was a kind gift from Dr. Pamela Robertson (Division of Molecular Medicine, University of Dundee). The HeLa cell line expressing Human Papillomavirus type 18 was from Cancer Research UK, Lincoln's Inn Fields, London, UK.

Dulbecco's Modified Essential Medium (DMEM) with high glucose and sodium pyruvate and heat inactivated Foetal Bovine Serum (HIFBS) were purchased from Invitrogen Life Science (3 Fountain Drive, Inchinnan Business Park, Paisley, UK PA4 9RF). Trypsin solution (2.5%) was purchased from Sigma Aldrich Company Ltd. (The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT). Tissue culture dishes and plastics were from Thermo Fisher Scientific (Thermo Fisher Scientific, Nunc A/S, Kamstruprej 90, P.O Box 280. DK-4000, Roskilde, Denmark). The Mistral 1000 centrifuge was procured from Measuring and Scientific Equipment (UK) Ltd (Worsley Bridge Road, Lower Sydenham, London, SE26 5AZ).

2.1.2 Method

All the cell lines were maintained in DMEM enriched with 10% HIFBS in 9 cm dishes. All culture incubations were performed in a humidified 5% CO_2 incubator at 37°C. When the cell lines had reached desired density they were washed with Phosphate-buffered saline (PBS) and detached from the surface of the culture dish using trypsin and centrifuge at 900 rpm for 5 min. The media was aspirated and the cell pellet was stored in a freezer at -20°C for use in genomic DNA extraction.

2.2 Extraction of genomic DNA from mammalian cell lines

2.2.1 Materials

The QIAGEN DNeasy Blood and Tissue Kit (Catalogue No. 69506) was a kind gift from Dr. David Edwards (Unit of Cell and Molecular Biology, Dundee Dental School) and was purchased from Qiagen (Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ). Molecular biology grade absolute ethanol (Catalogue No. BP2818-500) was purchased from Fisher-Scientific (Fair Lawn, New Jersey 07410). Phosphate Buffered Saline (PBS) tablets (Catalogue No. P4417-100TAB) was from Sigma-Aldrich Co. (3050 Spruce Street, St. Louis, MO 63103 USA).

2.2.2 Method

Each frozen cell pellet was allowed to thaw and genomic DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit according to the manufacturer's standard protocol. Briefly, the cell pellet was re-suspended in PBS (200 µl) and Proteinase K (20 µl). Buffer AL (200 µl) was added to the solution, mixed thoroughly with a vortex mixer for 10 sec followed by incubation at 56 °C in a water bath for 10 min. Next, ethanol (200 µl) was added to the solution and mixed. The solution was transferred to a DNeasy mini spin column placed in a 2 ml collection tube and centrifuged at ≥ 6000 x g for 1 min. After discarding the flow-through, Buffer AW1 (500 µl) was added to the spin column and centrifuged at ≥ 6000 x g for 1 min. Once again the flow-through was discarded and the DNeasy membrane was dried by adding Buffer AW2 to the spin column and centrifuging at ≥ 20,000 x g for 3 min. Finally, the DNA was eluted by placing the column in a clean collection tube and adding Buffer AE (200 µl) to the DNeasy membrane. The DNA concentration was measured and the DNA samples were stored at 2 – 4°C for downstream experiments.

2.3 Sectioning of formalin-fixed paraffin-embedded tissue specimens for genomic DNA extraction

2.3.1 Materials

The microtome HM 320 was from Microm UK Ltd. (Commerce House, Telford Road, Bicester OX26 4LD). The microtome blades, MB DynaSharp 34°/80mm (Product No. 12056679) was from Fisher Scientific Uk Ltd. (Bishop Meadow Road, Loughborough

LE11 5RG). Trial FFPE specimens of oral squamous epithelial tissue were a kind gift from Dr. Kevin Davey (Dundee Dental School). Trial FFPE specimen of normal oral tissue was a kind gift from Dr. Neil Kernohan (Department of Pathology, Ninewells Hospital). Archival tissue blocks of head and neck squamous cell carcinoma were provided by the Tayside Tissue and Tumour Bank (TTB) following an approval by the Tissue Bank Committee (Reference No. TR000325).

2.3.2 Method

FFPE specimens of three different sizes (0.1 cm^2, 0.5 cm^2 and 1 cm^2) were employed to compare protocols for DNA extraction and to determine the optimum thickness of tissue sections for downstream experiments. Whole tissue section curls (3 – 5) ranging from 5 - 10 µm thickness each were cut from each specimen for DNA extraction PCR.

Sectioning of archival FFPE specimens in this study was undertaken by Dr. Margaret Florence and Mrs. Valerie Wilson from Dundee Dental School. The archival tissue blocks were sectioned according to the standard laboratory protocol. H&E stained sections were prepared for each specimen prior to and following sectioning. Slides were inspected under the microscope to ensure the presence of tumour and the absence of tissue necrosis. Following optimisation of the DNA extraction protocol, five tissue section curls of 10 µm each were cut from archival tissue sample and collected in a 1.5 ml Eppendorf for DNA extraction. Appropriate precautions such as cleaning the microtome and using fresh blades between specimens were taken during sectioning to avoid inter-block DNA contamination was taken during sectioning by.

2.4 Genomic DNA extraction from formalin-fixed paraffin-embedded tissues

2.4.1 Protocol A: QIAamp DNA FFPE Tissue Kit

2.4.1.1 Materials

The QIAamp Formalin-fixed, paraffin embedded Tissue Kit (Catalogue No. 56404) was purchased from Qiagen (Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ). Xylene (Catalogue No. 1029364) was from BDH Prolabo ® (VWR International Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN England).

2.4.1.2 Method

Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit according to the manufacturer's standard protocol. Briefly, excess paraffin was removed from the tissue sections with Xylene (1 ml) and 96 – 100% ethanol (1 ml). The resulting cell pellet was re-suspended in Buffer ATL (180 µl) and Proteinase K (20 µl). The sample was incubated once at 56°C for 1 hour for sample lysis and again at 90°C for 1 hour. Following lysis, Buffer AL (200 µl) and 96 – 100% ethanol (200 µl) were added to the sample and mixed thoroughly using a vortex mixer. The lysate was transferred to a QIAamp MinElute column and centrifuged at 6000 x g for 1 min. After discarding the flow through, Buffer AW1 (500 µl) was added to the spin column and centrifuged at 6000 x g for 1 min. The flow through was discarded and Buffer AW2 (500 µl) was added to the column and centrifuged at 6000 x g for 1 min. Once again the flow thorough was discarded and the column was centrifuged at 20,000 x g for 3 min to dry the membrane. The column was transferred to a clean collection tube and the DNA was eluted in Buffer ATE (100 µl) by centrifuging the column at 20, 000 x g for 1 min. The DNA concentration was measured and the DNA samples were stored at -20°C in a freezer.

2.4.2 Protocol B: DNA Lysis Buffer (LC)

2.4.2.1 Materials

The Lysis Buffer (10 mM Tris HCL, 50 mM KCL, 2.5 mM $MgCl_2$, 0.45% Tween; pH balanced to 8.2) was a kind gift from Dr. Susan Bray (Tayside Tissue and Tumour Bank, Ninewells Hospital, Dundee) and is part of the standard laboratory protocol followed by the Tayside Tumour and Tissue Bank. The heat block DRI- BLOCK DB 2A was from Techne (Cambridge) Ltd. (Duxford, Cambridge CB2 4PZ).

2.4.2.2 Method

A working solution of the lysis buffer (400 µl) and proteinase K (4 µl) was prepared and kept on ice. Meanwhile, excess paraffin was removed from the tissue sections with xylene (1 ml) and 96 – 100% ethanol (1 ml). The working solution was added to the cell pellet and mixed thoroughly using a vortex mixer and the sample incubated in a water bath at 55°C for 2 hours. Next, the sample was centrifuged at 1000 x g for 1 min and

incubated at 95°C for 15 min. It was allowed to cool to room temperature before sodium acetate (3M, 0.1 volume) was added to the DNA sample and mixed using a vortex mixer. Ethanol (96 – 10%; 2 volumes) was added to the sample, mixed and incubated overnight in a freezer at -20°C. Next, the sample was centrifuged at 20,000 x g at 4°C for 15 min. The supernatant was discarded without disturbing the pellet and ethanol (70%; 500 µl) was added to the pellet. After incubation overnight at -20°C, the sample was centrifuged at 20,000 x g at 4°C for 2 min. The supernatant was discarded and the DNA air dried at room temperature. The DNA was dissolved in TE and stored at 2-4°C for use in subsequent experiments.

2.5 Determination of DNA concentration

2.5.1 Materials

NanoVue Plus™ spectrophotometer was from GE Healthcare Ltd. (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK).

2.5.2 Method

The DNA quantification was carried out according to the manufacturer's standard protocol. Before each DNA sample was measured, the spectrophotometer was calibrated using a reference sample – usually the buffer that the DNA was eluted into. With the sampling head lifted to a vertical position, a small amount of the DNA sample (2 µl) was pipetted carefully onto the bottom measurement plate. The sampling head was lowered and a recording was initiated according to the parameters set on the spectrophotometer. After each measurement was completed, the upper and lower plates were wiped clean using a soft laboratory tissue.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Materials

Techne TC-412 thermal cycler was purchased from Keison Prodcuts (P.O Box 2124, Chelmsford, Essex CM1 3UP, England). The Techne Progene thermal cycler was from Techne (Cambridge) Ltd. (Duxford, Cambridge CB2 4PZ).

Full length clones of HPV types 33 and 52 were employed as positive controls in

addition to DNA extracts from SiHa and Hela cell lines. DNA plasmids containing HPV 33 (HPV 33 in pBR322 at position 2797) and HPV 52 (HPV 52 in pUC19 at position 7559) were kindly supplied by Dr. Gerard Orth and Dr. Wayne Lancaster (International HPV reference Center, Karolinska Institutet, Stockholm). The consensus and type-specific oligonucleotides were purchased from Eurofins MWG Operon (Anzinger Str.7a, 85560 Ebersberg, Germany). MyTaq DNA polymerase (Catalogue No. BIO-21106) was purchased from Bioline Reagents Ltd. (Unit 16, The Edge Business Centre, Humber Road, London NW2 6EW).

2.6.2 Method

Working stocks of the oligonucleotides (20 µM) were prepared in 1 x TE buffer. Consensus and type-specific primers pairs employed in the amplification of various HPV related genes are described in Tables 2.1 and 2. 2

Primer	Oligonucleotide sequence (5' – 3')	Target gene	Nucleotide position on the genome	Amplicon length (bp)
GH20	GAAGAGCCAAGGACAGGTAC	Human β-globin	70400-70419	268 [251]
PC04	CAACTTCATCCACGTTCACC	Human β-globin	70648-70669	
MY09	CGTCCMARRGGAWACTGATC	HPV L1	7150-7170	450 [126]
MY011	GCMCAGGGWCATAAYAATGG	HPV L1	6722-6742	
CPI	TTATCA(T/A)ATGCCCA(T/C)TG TACCAT	HPV E1	1942-1964	188 [304]
CPIIG	ATGTTAAT(A/T)(G/C)AGCC (A/T)CCAAAATT	HPV E1	1777-1799	

Table 2.1 List of consensus primers employed in the detection and amplification of the Human β-globin gene, the HPV L1 and E1 genes. Oligonucleotide sequences of forward and reverse primers, their position on the genome and the PCR product sizes are also shown.

Primer	Forward (5' – 3')	Reverse (5' – 3')	Target gene	Nucleotide position on the genome	Amplicon length (bp)
Primer 343 & 344	GATGAAAT AGATGGTC CAGC	GCTTTGTAC GCACAACC GAAGC	HPV 16 E7	106 – 125 192 - 213	108 [313]
Primer 345 & 346	AAGAAAAC GATGAAAT AGATGGA	GGCTTCAC ACTTACAA CACA	HPV 18 E7	107 – 129 191 210	104 [313]
Primer 347 & 348	TGAGGATG AAGGCTTG GACC	TGACACAT AAACGAAC TGTG	HPV 33 E7	99 -118 189 -208	110 [313]
Primer 349 & 350	GCAGAACA AGCCACAA GCAA	TAGAGTAC GAAGGTCC GTCG	HPV 52 E7	139 – 158 243 - 224	105 [313]

Table 2.2 List of type-specific primers employed in the detection and amplification of the E7 genes of HPV 16, 18, 33 and 52. The oligonucleotide sequences of forward and reverse primers, their position on the genome and the PCR product sizes are also shown.

PCR was carried out in 50 µl reactions with 100 ng of template DNA prepared as described in Section 2.3. The master mix was made up as follows: extracted template DNA (100 ng), 5 x MyTaq Reaction Buffer (5 mM dNTPs, 15 mM MgCl2, stabilisers and enhancers) 10 µl, Forward primer (20 µM) 1µl, Reverse primer (20 µM) 1 µl, MyTaq DNA Polymerase 0.5 µl and ddH_2O upto 50 µl. The PCR reaction mixtures were placed in a thermal cycler and incubated for 3 min at 94^oC for DNA denaturation. Next, 35 cycles of amplification were performed with each cycle consisting of a denaturation step at 94^oC for 15 seconds, followed by primer annealing at the temperatures described in Table 2.3, and a chain elongation step at 72^oC for 15 seconds. A final elongation was performed at 72^oC for 5 min. Depending on the size of the amplicon, the PCR products were run on a 1%, 1.5% or 2% gels prepared as described in Section 2.7.

Primer pair	Annealing temperature	Time
GH20/PC04	58°C	15 seconds
MY09/MY011	50°C	15 seconds
CPI/CPIIG	52°C	15 seconds
Primer 343/344	54°C	15 seconds
Primer 345/346	54°C	15 seconds
Primer 347/348	56°C	15 seconds
Primer 349/350	56°C	15 seconds

Table 2.3 List of consensus and type-specific primers employed in the amplification and detection of the Human β-globin gene and various HPV-related genes along with their annealing temperatures and time.

2.7 Agarose gel electrophoresis

2.7.1 Materials

Molecular grade Ultra Pure™ Agarose (Catalogue No. 15510-027) was purchased from Invitrogen Life Science (3 Fountain Drive, Inchinnan Business Park, Paisley, UK PA4 9RF). The nucleic acid stain, Gel Red™ (Catalogue No.41003 -T) was purchased from Biotium, Inc. (3159 Corporate Place, Hayward, CA 94545). The DNA marker HyperLadder™ 100bp Plus and 5 X sample loading buffer (Catalogue No. BIO-33056) were purchased from Bioline Reagents Ltd. (Unit 16, The Edge Business Centre, Humber Road, London NW2 6EW). The running buffer 1 x TAE was prepared as in Appendix C.

The electrophoresis power supply units, EPS 3500/200, were from Pharmacia Biotech AB (S-751 82 Uppsala, Sweden). The Horizon 58 gel casting systems were from Life Technologies Ltd. (3 Fountain Drive, Inchinnan Business Park, Paisley UK). The imaging system used for visualisation of agarose gels, Bio-Rad CHEMI DOC™ MP (Catalogue No. 170-8280) was purchased from Bio-Rad Laboratories Ltd. Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX).

2.7.2 Method

Agarose gels (1%/1.5%/2%) were prepared by weighing out agarose (1 gm/1.5 gm/2 gm) in a conical flask. Buffer 1 x TAE (100 ml) was added to the agarose and the solution was heated in a microwave for 1 min. Meanwhile, a gel casting tank was prepared and an appropriate sized comb was inserted. When the agarose had completely dissolved, Gel Red stain (6 µl) was added and the cooled solution was poured into the prepared casting tank. The gel was allowed to set before topping with 1x running buffer TAE. Meanwhile, DNA samples were prepared for loading by mixing the PCR products (5 µl) with loading dye (2 µl). When the gel was ready the comb was removed, the DNA samples and a standard ladder loaded into the wells of the gel and electrophoresed at 80V for 50 min. The DNA bands were visualised using the Bio-Rad gel imaging system.

2.8 Purification of PCR products for DNA sequencing

2.8.1 Materials

QIAquick Gel Extraction Kit (Catalogue No. 28704) was purchased from Qiagen (Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ). Isopropanol was purchased from BDH Prolabo ® (VWR International Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN England).

2.8.2 Method

PCR products were purified using the QIAquick Gel Extraction Kit according to the manufacturer's standard protocol. Briefly, the DNA fragments from the agarose gel were excised and weighed in a colourless tube. The agarose was solubilised with the addition of Buffer QG (3 volumes buffer: 1 volume gel) followed by incubation at 50°C for 10 min. Next, Isopropanol (1 gel volume) was added to the sample and mixed thoroughly. The sample was transferred to a QIAquick spin column and centrifuged for 1 min at 17,900 x g. The flow through was discarded and Buffer PE (0.75 ml) was added to the column and centrifuged for 1 min at. Next, the 17,900 x g flow through was discarded and the DNA was eluted by adding de-ionised H_20 (50 µl) to the spin column.

2.9 DNA sequencing

DNA sequencing of PCR products was undertaken by Dr. Andrew Cassidy, Tayside Centre for Genomic Analysis, Ninewells Hospital. The protocol is briefly outlined below. The PCR products were purified using a modification of the ExoSAP enzymatic clean-up method. The PCR products (5 µl) were incubated with 1 U of exonuclease I and 1 U of shrimp alkaline phosphatase for 20 min at 37°C then inactivated by incubating at 80°C for 15 min. Samples were sequenced bidirectionally using the ABI BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) and analysed on the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). Sequence analysis was performed using MacVector version 12.03 (MacVector Inc., Waterbeach, Cambridge, UK) [231].

2.10 Immunohistochemistry

2.10.1 Materials

The microtome HM 320 was from Microm UK Ltd. (Commerce House, Telford Road, Bicester OX26 4LD). The microtome blades, MB DynaSharp 34°/80mm (Product No. 12056679) was from Fisher Scientific Uk Ltd. (Bishop Meadow Road, Loughborough LE11 5RG). The procurement of trial FFPE specimen of tonsil tissue and archival tissue specimens for the study is described in Section 2.3.1.

SuperFrost ® Plus Adhesion slides (Catalogue No. 631-9483) were from Thermo Fisher Scientific Inc. (VWR International Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN England). Fraction V, Heat shock treated Bovine Serum Albumin (Catalogue No. BPE-1600-100) and clearing agent. Hydrogen Peroxide solution, 30% (w/w) was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Riedstr, 2 D-89555 Steinheim 49 7329 970). The EBP50 polyclonal primary antibody (Catalogue No. PA1-090) was from Pierce Antibody (Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leics, LE11 5RG, UK). Vectastain ABC kit was purchased from Vector Laboratories (3 Accent Park, Bakewell Road, Orton Southgate, Peterborough PE2 6XS). Diaminobenzidine was from Sigma Aldrich Company Ltd and DePeX mounting medium was from VWR International Ltd. The bacterial protein lysates of GST and GST-EBP50 were a kind gift from Dr. Sam Crouch (Department of Oral and Maxillofacial Clinical Sciences, Dundee Dental School).

2.10.2 Method

2.10.2.1 Tissue sectioning and preparation of slides

Sectioning of archival tissue specimens for immunohistochemical analysis was undertaken by Dr. Margaret Florence from Dundee Dental School. Sections of 5 – 6 µm thickness were cut using a microtome from each tissue specimen. The sections were transferred onto Super Frost plus slides. This was followed by heating the slides at 60^{o}C for 20 min in an oven to allow for attachment of the tissue and melt the paraffin. The slides were stored at room temperature.

2.10.2.2 Antigen retrieval

Immunohistochemistry of archival specimens for this study was undertaken by Athiva Shankar with technical assistance from Dr. Margaret Florence. The sections were prepared for antigen retrieval by de-paraffinising in two 5 min washes with the clearing agent xylene. This was followed by 2 min washes in graded ethanol solutions of 100%, 90% and 70% to re-hydrate the tissue sections. The sections were rinsed with dH_20. Meanwhile, 10 mM citrate buffer (pH 6.4) was preheated for 15 min under microwave pressure. The sections were gently warmed before being transferred to the preheated citrate buffer followed by heating for 15 min under microwave pressure. The sections were allowed to cool for 10 min before washing in tap water.

2.10.2.3 Immunohistochemistry

Next, the sections were incubated in 10% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Following hydrogen peroxide incubation, the sections were washed thoroughly in tap water followed by rinsing with PBS. Immunoperoxidase staining was performed using the Vectastain ABC kit according to the manufacturer's instructions. Briefly, the non-specific binding sites on the tissue sections were blocked with normal goat serum (diluted to 1:65 in 1% BSA/PBS (w/v) for 20 min. This was followed by incubation with primary antibody diluted to appropriate concentration (PA1-090 Anti-EBP50; 1:2000) in 1% BSA/PBS (w/v) for 30 min at room temperature in a humidified atmosphere. The sections were washed (three 5 min washes) with wash buffer to remove unbound antibody followed by incubation with biotinylated secondary antibody diluted 1:200 in 1% BSA/PBS (w/v) for 30 min in a humidified atmosphere. Meanwhile, Vectastain ABC reagent was prepared by diluting Reagent A 1:50 in 1% BSA/PBS (w/v) and adding to Reagent B also at a dilution of 1:50 and allowed to stand

for 30 min. The sections were incubated for 30 min with the prepared ABC reagent after three 5 min washes in wash buffer and rinsing in PBS. Excess ABC reagent was removed from the slides by washing with wash buffer and rinsing in PBS. The sections were incubated in peroxidase substrate solution for 5 - 8 min and washed in tap water. The sections were counterstained with haematoxylin dye and dehydrated by immersing in graded ethanol solutions of 70%, 90% and 100% for 2 min each followed by clearing in xylene (two 5 min incubations). Tissue sections were mounted with DePeX mounting medium using coverslips and allowed to dry before visualising under a light microscope (Motic BA400)**.** Images were captured at x100 or x400 magnification (scale bar = 1 cm).

2.10.2.4 Antibody blocking

The primary antibody, anti-EBP50 was combined with GST (2 µg, 5 µg), GST-EBP50 (2 µg, 5 µg) or no bacterial proteins and incubated on ice for 20 min or overnight at 2-4^{o}C. The blocked antibody and controls were applied to the tissue sections as for routine immunohistochemistry.

2.10.2.5 Scoring of EBP50 immunohistochemistry results

The protocol for scoring of EBP50 immunohistochemistry results for this study was adapted from Xiao-Guang *et al*.[183]. The sections were analysed by two independent observers (Athiva Shankar and Dr. Sam Crouch) blinded to the sample identities. Additionally, 10% of the sections were scored by a third independent observer (Dr.Michaelina Macluskey). Differences in inter-observer scores were reconciled through discussions and repeat viewing of the sections. Five random representative fields of 100 cells each were viewed using x 40 magnification. Based on the percentage of positively stained tumour cells, membrane and cytoplasmic EBP50 expression were scored separately as follows:

Membrane staining

Negative – No staining in >10% of the tumour cells

Positive – Weak or moderate staining in >10% of the tumour cells

Mixed – Combination of the above

Cytoplasmic staining

Negligible/Weak - Negligible/Weak staining in >10% of the tumour cells

Positive - Moderate staining in >10% of the tumour cells

Strong - Marked staining in >10% of the tumour cells

Mixed – Combinations of the above

Each specimen was assigned a final score to reflect membrane and cytoplasmic EBP50 expression patterns.

2.11 *In Situ* Hybridisation

In Situ Hybridisation (ISH) was undertaken by the Department of Clinical Pathology, Ninewells Hospital. Briefly, sections of 4 μm thickness were cut and mounted on SuperFrost Plus slides. The INFORM HPV III probe sets capable of detecting 13 oncogenic HPV types (Ventana Medical Systems) was employed in the assay. The ISH assay was performed using the BenchMark automated slide staining system (Ventana Medical System) according to the manufacturer's guidelines. The slides were scored separately by two independent observers (Dr. Sharon White, Department of Pathology, Ninewells Hospital and Athiva Shankar). Punctate nuclear staining was considered a positive result for HPV DNA**.**

2.12 Patient selection and clinical data collection

This study was approved by the Caldicott Guardian and the Tayside Tissue Bank and conducted according to the guidelines outlined by the Research Governance Framework in Tayside, Scotland.

Patients were selected, based on the availability of a p16 status, from an existing head and neck database with HNSCC patients treated between January 2006 and December 2011 in NHS Tayside, Scotland. The parent database was developed as part of a previous study and involved combining two clinician-led datasets including information on smoking and drinking behaviours at diagnosis held in the Head & Neck unit as well as electronic case note reviews to identify any missing clinical (e.g. treatment) and pathological (e.g. tumour staging, margins, histological type, grade of differentiation, invasive front) data. The p16 status of the patient samples was determined by immunohistochemistry. The slides were analysed by a single pathologist (Dr. Sharon White) in the Department of Pathology, Ninewells Hospital and were scored as positive

if strong diffuse nuclear and cytoplasmic staining was observed in ≥ 70% of the tumour [282].

Following selection of patients based on p16 status, the database for this study was updated with survival data and clinical information where it was missing by reviewing case notes. Survival outcomes were assessed against p16 status, smoking and alcohol history, disease stage, HPV status and EBP50 expression. A completed database was transferred to Tayside Health Informatics Centre (HIC) for anonymisation via a secure NHS transfer system. For immunohistochemical analysis of EBP50 expression, a smaller sub-cohort was selected against the HPV status of the patients.

2.13 Statistical analysis

In this dataset, all clinical data related to age, diagnosis, treatment, smoking and alcohol history was coded according to a coding manual developed for the original database. Primary tumour sites were categorised according to the Royal College of Pathologists-Datasets for histopathology reporting of mucosal malignancies of the head and neck [300]. Smoking [63] and alcohol were classified using case note information, as follows:

- Light smoker - 1-14/day
- Moderate smoker - 15-24/day
- Heavy smoker - ≥ 25/day
- Light to moderate drinker - 'no history of alcohol abuse' or 'moderate drinker'.
- Heavy drinker - alcohol consumption of ≥ 21 units/week in men and ≥ 14 units/week in women (where figures were available in case notes) or 'excessive alcohol consumption'
- Ex- heavy drinker - 'history of alcohol abuse or heavy drinking'

Data was analysed using SPSS package (IBM Statistics Version 22). Data related to categorical variables was described in terms of number of patients (percentages) and as mean or median for continuous variables. Chi-squared test of independence was used to report on pairwise correlation between p16 status, HPV status and EBP50 expression on the one hand and social demographics and clinical/histopathological characteristics of the cohort on the other. Statistical significance was defined as $p < 0.05$. The Likelihood

Ratio test was used to compare the relative strength of p16 and EBP50 expression as indicators of HPV infection. Overall Survival (OS) was defined as time (in months) from diagnosis to death, end of study or date of last follow-up. Recurrence Free Survival (RFS) was defined as time (in months) from diagnosis to locoregional recurrence. Disease Free Survival (DFS) was defined as time (in months) from diagnosis to death due to head and neck cancer. Kaplan-Meier analysis was used to obtain 2-year and 5-year survival curves. The Cox Proportional hazards model was used to estimate Hazard Ratio (HR) and 95% Confidence Interval (CI) for prognostic significance of single and multiple variables.

2.14 Contents of laboratory kits

QIAGEN DNeasy Blood and Tissue Kit (Catalogue No. 69506)

- Buffer ATL
- Buffer AL
- Buffer AW1
- Buffer AW2
- Buffer AE
- Proteinase K
- DNeasy mini spin columns in 2ml collection tubes
- Collection tubes (2ml)
- Handbook

QIAamp DNA FFPE Tissue Kit (Catalogue No. 56404)

- Buffer ATL
- Buffer AL
- Buffer AW1
- Buffer AW2
- Buffer ATE
- Proteinase K
- QIAamp MinElute ® Columns
- Collections tubes (2ml)
- Handbook

QIAquick Gel Extraction Kit (Catalogue No. 28704)

- QIAquick spin columns
- Buffer QG
- Buffer PE
- Buffer EB
- Collection tubes (2ml)
- Loading buffer
- Handbook

MyTaq DNA Polymerase

- MyTaq DNA Polymerase 2 X 250µl
- 5 x MyTaq Reaction Buffer 14 x 1.5ml

2.15 General laboratory equipment and consumables

The water bath was from Grant Instruments (Cambridge) Ltd. (29 Station Road, Shepreth, Royston, Herts SG8 6P2 England). The vortex mixer, Vortex Genie 2 was purchased from Scientific Industries Inc. (70 Orville Drive, Bohemia, New York 11716 USA). The micro-centrifuge 5415D and the PCR tubes were purchased from Eppendorf North America Inc. (1 Catiague Road, P.O.Box 1019, Westbury, NY 11590 -0207). The Heraeus™ Biofuge™ Stratos™ benchtop centrifuge for ethanol precipitation was from Thermo Scientific UK Ltd. (Bishop Meadow Road, Loughborough, Leicestershire LE115RG). The regular pipette tips were from Sarstedt Ltd. (68 Boston Road, Beaumont Leys, Leicester LE4 1AW, UK). The filter pipette tips used for PCR were purchased from Axygen BioScience, Inc. (33210 Central Avenue, Union City CA 94587). The pipettes were from Gilson, Inc. (3000 Parmenter Street, P.O Box 620027 USA). The Eppendorf's were from Alpha Laboratories Ltd. (40 Parham Drive, Eastleigh, Hampshire SO50 4NU).

2.16 Solutions and Buffers

3M Sodium Acetate pH 5.2

Sodium acetate

dH_2O

Adjust pH to 5.2 with glacial acetic acid and adjust volume with dH_2O

Phosphate-buffered Saline

PBS tablets

dH_2O

1 x TAE Buffer

Tris acetate pH 8.2

EDTA

1 x TE Buffer

Tris HCl pH 7.5

EDTA

Chapter 3

A DESCRIPTIVE PROFILE OF THE COHORT

3.1 Selection of the sample population

NHS Scotland comprises 14 regional NHS Boards (Fig. 3.1(A)), 7 special NHS Boards and 1 public health body. Together they are responsible for the improvement of the health of the Scottish population by providing a range of healthcare and specialist services [223].

The sample population for this study includes patients diagnosed and treated for HNSCC in NHS Tayside. The health board of NHS Tayside covers an area of 7,508 sq. km encompassing the three local areas of Dundee City, Angus and Perth & Kinross with an estimated population of around 416,934 persons [224], [7]. On an average, 75 to 80 new cases of head and neck cancers are registered in Tayside every year. The focus of this study is Tayside, which is demarcated in black in Figure 3.1(A).

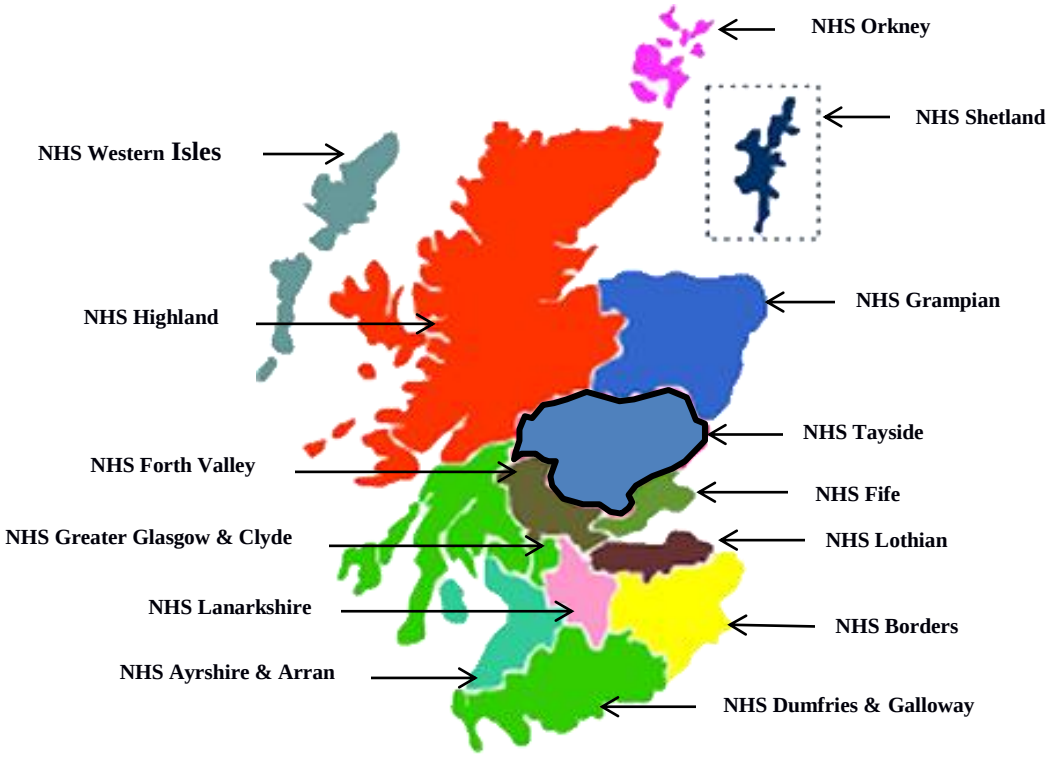

Figure 3.1 (A): Regional NHS Boards in Scotland. Fourteen regional NHS Boards constitute NHS Scotland along with 7 special boards and 1 public health body. The study population was selected from NHS Tayside (outlined in black).

At the beginning of the study 436 patients were selected from an existing database of HNSCC patients who were diagnosed and treated in NHS Tayside between the years 2006 to 2011. The availability of a p16 status for each patient was a key inclusion criterion. Patients were later excluded from the cohort at three subsequent stages:

where tissue specimens were unavailable for analysis, where specimens were assessed as being unsuitable for downstream experiments and where extracted DNA from the tissue specimens was found to be of poor quality. This meticulous selection of tissue specimens ensured a robust, yet large, working sample size of 293 patients. Figure 3.1 (B) is a detailed schematic representation of the patient selection process for the study.

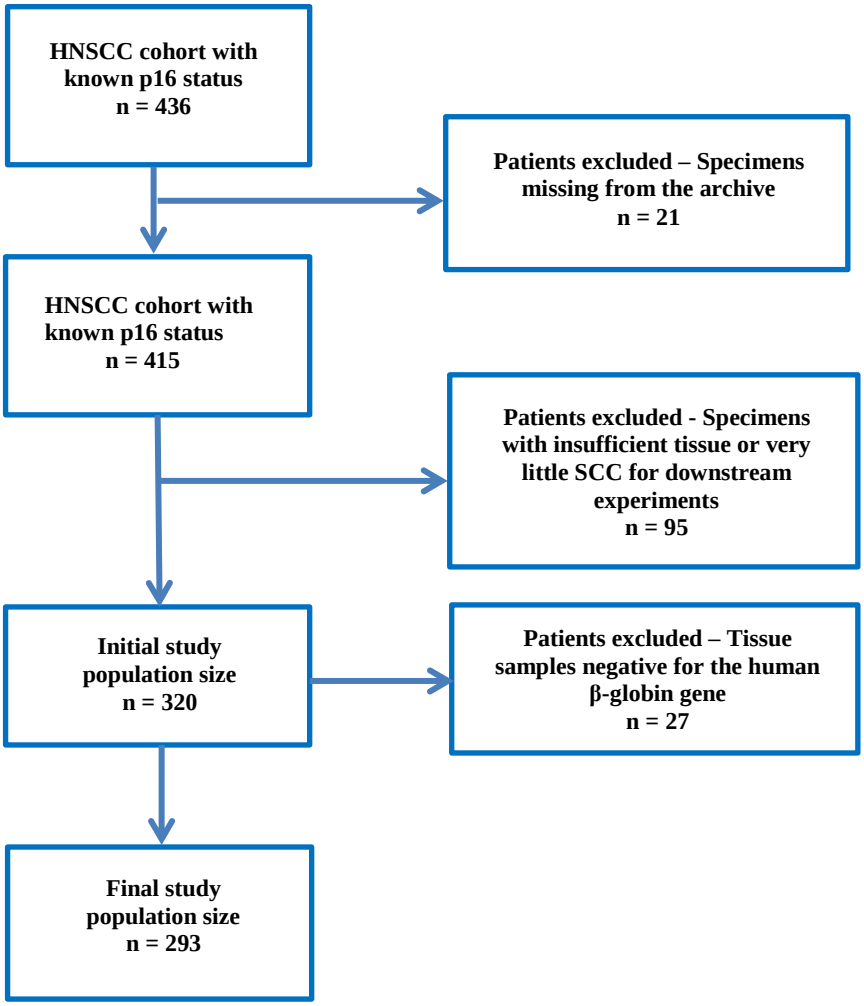

Figure 3.1 (B): Schematic representation of patient selection process. An existing database of HNSCC patients was screened and those with a definitive p16 status were included in the initial study cohort (n = 436). The initial cohort was filtered at 3 subsequent stages to exclude patients with missing biopsy specimens (n = 21), those with tissue specimens deemed unsuitable for downstream experiments (n = 95) and lastly, those patients for whom the quality of DNA extracted from tissue specimens was found to be questionable (n = 27). After exclusion of 143 patients, the final study cohort contained 293 patients.

3.2 Social demographics of the study population

A description of the study population based on social demographics is outlined in Table 3.1. More than half of the patient population was male (68%). Patients were categorised into 4 distinct age groups: ≤ 58 years (31%), 59 – 64 years (21%), 65 – 73 (25%) and ≥ 74 years (23%). The mean age at diagnosis was 65 years.

History of tobacco smoking and alcohol consumption was classified based on self-reported case note information as follows:

- Light smoker – 1 - 14/day

- Moderate smoker – 15 - 24/day
- Heavy smoker – ≥ 25/day
- Light to moderate drinker - 'no history of alcohol abuse' or 'moderate drinker'
- Heavy drinker - alcohol consumption of ≥ 21 units/week in men and ≥ 14 units/week in women (where figures were available in case notes) or 'excessive alcohol consumption'
- Ex- heavy drinker - 'history of alcohol abuse or heavy drinking'

More than one-third of the patient population were smokers at the time of diagnosis and while the majority were 'Light to moderate drinkers' (40%), nearly one-quarter had a history of heavy drinking (current or ex- heavy drinker).

Characteristic	No of patients (n = 293)	No of patients (%)
Gender		
Male	198	68
Female	95	32
Age at diagnosis (Groups)		
≤ 58	92	31
59 – 64	61	21
65 – 73	72	25
≥ 74	68	23
Mean age at diagnosis	**64.5 (Range 30 – 90)**	
History of smoking at diagnosis		
Non – smoker	47	16
Light smoker	23	8
Moderate smoker	50	17
Heavy smoker	40	14
Ex- smoker	74	25
Other	11	4
Data missing or unavailable	48	16
History of alcohol consumption at diagnosis		
Non-drinker	34	12
Light to moderate drinker	117	40
Heavy drinker	71	24
Ex- heavy drinker	9	3
Data missing or unavailable	62	21

Table 3.1: Profile of study population based on social demographics. Patients were categorised into 4 distinct age groups with the mean age at diagnosis being 65 years. More than one-third of the study cohort were smokers at the time of diagnosis and nearly a quarter had a history of heavy drinking.

3.3 Pathological features of tumours and disease staging

Tables 3.2 (A) and 3.2 (B) summarise the profiles of the study population based on tumour site, pathological features and disease stage. Site of primary tumour was categorised according to the Royal College of Pathologists Dataset for histopathology reporting of mucosal malignancies of the head and neck [300]. Tumours were grouped into those arising in the oral cavity, oropharynx, larynx, hypopharynx, unknown primary and other less common sites. While oral cavity and laryngeal tumours constituted 34% and 30% respectively, oropharyngeal tumours accounted for 21% of the cohort. Unknown primaries, tumours arising in the hypopharynx and other less common sites (pharynx, nasopharynx, nasal cavity and maxillary sinus) accounted for

the remaining 15%. The vast majority of tumours were either moderately differentiated (41%) or poorly differentiated (39%). Neck nodes involved by carcinoma was noted in 143 (49%) patients and extracapsular spread, a manifestation of biological aggressiveness of tumours, was noted in 48 (16%) patients. Additional histopathological features of prognostic value such as the presence of a non-cohesive invasive front, lymphovasular and perineural invasion were also documented. While presence of a non-invasive cohesive front was noted in 71 (24%) patients, lymphovasular invasion (12%) and perineural invasion (7%) were recorded only in a small percentage of the patient population. It is important to note here that the histopathological parameters of invasive front, lymphovasular and perineural invasion had significant proportions of missing data.

Like most cancers, prognosis in mucosal cancers of the head and neck largely depends on tumour stage. The TNM staging system describes the anatomic extent of the primary tumour along with the involvement of regional lymph nodes and distant metastases, if any [298]. A significant proportion of patients in the cohort had histologically confirmed T4 stage (31%). The percentage of study population with T2, T1 and T3 was 25%, 20% and 16% respectively. Nodal involvement at N2 stage was noted in more than a quarter of the study population. At the time of diagnosis nearly half the study population (47%) had Stage IV disease (UICC Staging). Disease stages I, II and III were recorded at 16%, 14% and 15% of the cohort respectively.

Characteristic	No of patients (n = 293)	No of patients (%)
Site of primary tumour		
Oral cavity	99	34
Oropharynx	60	21
Larynx	88	30
Hypopharynx	21	7
Unknown primary	9	3
Other	15	5
Grade of differentiation		
Well differentiated	5	2
Moderately differentiated	119	41
Poorly differentiated	115	39
Moderate – Poorly differentiated	28	10
Other	12	4
Data missing or unavailable	14	4

Neck node status		
Negative	146	50
Positive	143	49
Unknown	4	1
Extracapsular spread		
Negative neck node status	148	50
Yes	48	16
No	58	20
Inconclusive	2	1
Data missing or unavailable	37	13
Non-cohesive invasive front		
Yes	71	24
No	107	37
Not applicable – Unknown primary	10	3
Data missing or unavailable	105	36
Lymphovascular invasion		
Yes	36	12
No	142	48
Not applicable – Unknown primary	11	4
Inconclusive	2	1
Data missing or unavailable	102	35
Perineural invasion		
Yes	20	7
No	122	42
Not applicable – Unknown primary	12	4
Data missing or unavailable	139	47

Table 3.2 (A): Profile of study population based on anatomic site of primary tumour, grade of tumour differentiation and other pathological traits. The tumours in this cohort involved mainly the oral cavity, the larynx and the oropharynx. The vast majority of them were either moderately differentiated (41%) or poorly differentiated (39%). Neck node involvement by tumours was noted in 49% of the cohort. Histological features for tumour aggression such as the presence of extracapsular spread (16%), a non-cohesive invasive front (24%), lymphovascular invasion (12%) and perineural invasion (7%) were also noted although a large proportion of clinical data pertaining to the latter was missing.

Variable	No of patients (n = 293)	No of patients (%)
TNM Classification		
T (Tumour) Stage		
T1, T1a, T1b	58	20
T2, T2a, T2b	74	25
T3	48	16
T4, T4a, T4b	90	31
TX	8	3
T stage data missing	15	5
N (Node) Stage		
N0	135	46
N1	42	14
N2, N2a, N2b, N2c	93	32
N3	8	3
N stage data missing	15	5
UICC Staging		
Stage I	47	16
Stage II	42	14
Stage III	43	15
Stage IVA	131	45
Stage IVB	7	2
Unknown	23	8

Table 3.2 (B): Profile of study population based on TNM Classification and UICC staging. Nearly half of the study population had presented with late stage disease at the time of diagnosis.

3.3 Main therapeutic interventions in the treatment of the sample population

Surgery is a standard treatment for HNSCC but is often limited by anatomical extent of the tumour and the physiological role of involved structures. The need to improve survival outcomes and preserve organ function has necessitated the use of multi-modal treatment strategies involving a combination of surgery, chemotherapy and/or radiotherapy [85]. Table 3.3 catalogues the main therapeutic interventions employed in the treatment of patients in the cohort, including chemotherapy regimen and radiotherapy dose. The sample population had undergone either single modality treatment in the form of radiotherapy (25%) and surgery (19%) or combined modality treatment in the form of chemoradiation (18%), surgery with chemoradiation (15%) and surgery with radiotherapy (11%). A small number of patients (n = 23; 8%) received only palliative care. A significant percentage of patients treated surgically had neck dissection (21%). Cisplatin, either alone (7%) or in combination with Fluorouracil

(5FU) (23%), was the drug of choice for chemotherapy with regimens administered neo-adjuvantly and/or concurrently. Radiotherapy dose and fractions were administered as 60-68 Gy in 30-34 fractions (31%) and 50-55Gy in 20 fractions (27%).

Treatment	No of patients (n = 293)	No of patients (%)
Treatment modalities		
Surgery alone	54	19
Radiotherapy alone	74	25
Chemotherapy +/- Surgery	7	2
Chemoradiation	53	18
Surgery, Chemotherapy and Radiotherapy	45	15
Surgery and radiotherapy	32	11
No treatment/Palliative care	23	8
Data missing or unavailable	5	2
Type of surgery		
Surgery to primary only	33	11
Surgery to primary and neck dissection	62	21
Neck dissection only	37	13
Surgery not a treatment modality	156	53
Data missing or unavailable	5	2
Chemotherapy – Drugs		
Cisplatin/5FU	68	23
Cisplatin	20	7
Other	16	5
Chemotherapy not a treatment modality	184	63
Data missing or unavailable	5	2
Chemotherapy – Regimen		
Neo-adjuvant	20	7
Concurrent	23	8
Neo –adjuvant + concurrent	56	19
Other	5	2
Chemotherapy not a treatment modality	184	63
Data missing or unavailable	5	1
Chemotherapy – Cycles		
1 -3	46	16
4 – 6	51	17
7 - 8	7	3
Chemotherapy not a treatment modality	184	63
Data missing or unavailable	5	1
Radiotherapy – dose and fractions		
60 – 68 Gy in 30 – 34 fractions	91	31
50 – 55 Gy in 20 fractions	78	27
40 – 44.5 Gy in 12 & 16 fractions	18	6
Radiotherapy not a treatment modality	85	29
Data missing or unavailable	21	7

Table 3.3: Profile of study population based on main therapeutic interventions employed in the treatment of HNSCC. The patients had undergone either single modality treatment in the form of radiotherapy or surgery or combined modality treatment in the form of chemoradiation, surgery with chemoradiation or surgery with radiotherapy. A significant percentage of patients treated surgically had undergone neck dissection. Chemotherapy regimens were administered neo-adjuvantly and/or concurrently with Cisplatin, either alone or in combination with Fluorouracil (5FU) being the drug of choice. Radiotherapy dose and fractions were administered as 60-68 Gy in 30-34 fractions and 50-55Gy in 20 fractions.

3.5 Mortality and recurrence in the sample population

The mean length of follow-up, defined as the date of diagnosis to the date of last follow up, end of study period or the date of death, was 39 months (3+ years). However, only around three-fourths of patient population was followed up for 60 months (5 years).

At the end of the study period, 149 patients (51%) were deceased, 134 (46%) were alive disease free and 10 (3%) alive with disease. Out of 149 deaths, 58 (20%) were attributed to head and neck cancer and 42 (14%) to other causes. Cause of death data was unavailable for 49 patients (17%). Recurrence of primary disease was noted only in a small proportion of patients (19%) with commonly afflicted anatomic sites being the oral cavity (16/55; 29%), lymph nodes (15/55; 27%) and the larynx (12/55; 25%). Recurrence in the oropharynx (3/55; 6%) was relatively low (Table 3.4).

Variable	No of patients (n = 293)	No of patients (%)
End of study – Overall mortality	149	51
Cause specific mortality		
HNC	58	20
Other	42	14
Cause of death unknown	49	17
End of study – Alive with disease	10	3
End of study – Alive disease free	134	46
Recurrence		
Yes	55	19
No	231	79
Data missing or unavailable	7	2
Site of recurrence		
Nodal disease	15	27
Oral cavity	16	29
Larynx	12	22
Oropharynx	3	6
Other	9	16

Table 3.4: Profile of study population based on overall mortality and recurrence of disease. At the end of the study period, 49% of the cohort was alive and 51% was deceased. Only 20% of the deaths were attributed to head and neck cancer. Recurrence of primary disease was noted in 19% of the patients with commonly afflicted anatomic sites being the oral cavity, lymph nodes and the larynx.

The mean Overall Survival (OS) for the cohort was 58 months and 2-year OS was at 62% (Table 3.5). On univariate analysis, age of the patients, smoking history, site of primary tumour, disease stage, neck node status, extracapsular spread and treatment modality were found be significantly associated with OS (Table 3.5). Overall survival was significantly improved for patients in the age group of 59 to 64 or younger ($P < 0.0001$). Patients who were smokers at the time of diagnosis had a worse prognosis when compared to non-smokers and ex-smokers ($P = 0.002$). Patients presenting with early stage disease ($P < 0.0001$) and with tumours arising in the oropharynx and larynx ($P = 0.017$) were likely to survive longer. Positive neck nodes ($P < 0.0001$) and presence of extracapsular spread ($P < 0.0001$) were associated with poor clinical outcomes. Treatment modality also significantly influenced 2-year overall survival with patients who were treated by radiotherapy as a single treatment modality or in combination with surgery or chemotherapy having the worst prognosis. Multivariate analysis of the cohort identified smoking history (hazard ratio, HR 3.28, 95% confidence interval, CI 1.81 – 9.12, $P = 0.023$), extracapsular spread (hazard ratio, HR 0.25, 95% confidence interval, CI 0.1 – 0.63, $P = 0.004$) and treatment modality (hazard ratio, HR 15.1, 95% confidence interval, CI 2.29 – 100.2, $P = 0.005$) as independent prognostic variables for OS.

The mean Recurrence Free Survival (RFS) for the cohort was 72 months and 2-year RFS was at 79% (Table 3.5). On univariate analysis, age, smoking history, site of primary tumour, disease stage and treatment modality were not found to be significantly associated with RFS.

The mean Disease Specific Survival (DSS) for the cohort was 85 months and 2-year DSS was at 82 % (Table 3.5). On univariate analysis, smoking history, site of primary tumour, neck node status and extracapsular spread and treatment modality were found be significantly associated with DSS (Table 3.5). As expected, non-smokers and ex-smokers had a better 2-year DSS than current smokers ($P = 0.018$). Oropharyngeal and laryngeal tumours were associated with improved survival ($P = 0.017$). Positive neck nodes ($P < 0.0001$) and presence of extracapsular spread ($P < 0.0001$), both features of tumour aggression, were associated with poor outcomes. Treatment modality also significantly influenced 2-year DSS. Patients who had undergone chemoradiation and radiotherapy as a single treatment modality had the worst prognosis. However, none of

the aforementioned clinicopathological features were identified as independent prognostic factors for DSS following a multivariate analysis of the cohort.

Clinicopathological characteristics	# (patients, events)	Mean OS (month)	2-year OS (%)	P-value	# (patients, events)	Mean RFS (months)	2-year RFS	P-value	# (patients, events)	Mean DSS (months)	2-year DSS	P-value
All patients	(293,149)	58	62	-	(293, 55)	72	79	-	(293, 62)	85	82	
Age												
<58	(92, 32)	74	75		(87, 16)	75	86		(92, 14)	90	87	
59 – 64	(61, 25)	55	74	<0.0001	(55, 9)	67	83	0.274	(61, 13)	67	86	0.215
65 – 73	(72, 45)	46	56		(58, 16)	61	72	(NS)	(72, 16)	76	77	(NS)
>74	(68, 47)	38	41		(57, 14)	61	68		(68, 15)	71	75	
History of smoking												
Non-smoker	(47, 13)	74	77		(42, 7)	71	84	0.424	(47, 5)	86	88	
Current smoker	(124, 71)	50	62	0.002	(111, 24)	71	78	(NS)	(124, 31)	73	80	0.025
Ex-smoker	(74, 27)	63	69		(68, 10)	76	85		(74, 9)	82	87	
Site of primary tumour												
Oral cavity	(99, 52)	53	60		(89, 25)	65	70	0.177	(99, 23)	73	78	
Oropharynx	(60, 25)	56	68	0.017	(55, 7)	72	86	(NS)	(60, 10)	75	83	0.024
Larynx	(88, 41)	64	69		(81, 17)	68	84		(88, 12)	93	93	
Other	(46, 31)	38	48		(32, 6)	65	74		(46, 13)	61	69	
Neck node status												
Negative	(146, 60)	69	75	<0.0001	(139, 29)	74	82	0.339	(146, 25)	88	90	0.025
Positive	(143, 86)	44	51		(114, 25)	62	76	(NS)	(143, 32)	75	73	

Extracapsular spread												
Yes	(48, 34)	-	36		(43, 11)	-	65	0.105	(48, 18)	-	53	
No	(58, 23)	-	71	<0.0001	(50, 8)	-	87	(NS)	(58, 6)	-	90	<0.0001
Inconclusive	(2, 0)	-	100		(2, 0)	-	100		(2, 0)	-	100	
Disease stage												
Stage I	(47, 12)	86	89		(47, 11)	73	84		(47, 5)	100	95	
Stage II	(42, 17)	70	81		(41, 10)	60	79	0.346	(42, 8)	84	90	
Stage III	(43, 21)	52	70	<0.0001	(40, 5)	66	88	(NS)	(43, 7)	69	87	0.015
Stage IVA & IVB	(138, 85)	45	45		(110 , 20)	65	72		(138, 34)	71	72	
Unknown	(9, 4)	54	78		(8, 1)	62	83		(9, 1)	65	89	
Treatment modality												
Surgery alone	(54, 17)	80	82		(54, 11)	75	83		(54, 6)	99	94	
Radiotherapy alone	(74, 44)	50	60		(71, 18)	62	78		(74, 18)	76	79	
Chemotherapy +/- Surgery	(7, 6)	6	0	<0.0001	(5, 1)	2	50	0.046	(7, 4)	7	0	
Chemoradiation	(53, 26)	54	66		(51, 10)	69	76	(NS)	(53, 13)	70	81	<0.0001
Surgery, Chemotherapy and Radiotherapy	(45, 12)	70	78		(44, 6)	71	85		(45, 5)	81	88	
Surgery and Radiotherapy	(32, 16)	55	69		(32, 9)	52	70		(32, 5)	83	86	

Table 3.5: Univariate analysis related to Overall Survival, Recurrence Free Survival and Disease Specific Survival in the study cohort. 2-year Overall and cause- specific survival of the study cohort was significantly associated with smoking history, site of primary tumour, disease stage, neck node status, extracapsular spread and treatment modality. While age appeared to be an important prognostic indicator for overall survival, no statistically significant associations were noted for disease specific survival. None of the aforementioned factors were found to be predictors of recurrence free survival in this study cohort.

3.6 Discussion

The incidence of squamous cell carcinomas of the head and neck in Scotland has increased by 10.7% from 2004 to 2014 afflicting approximately 1234 patients per annum [128]. Furthermore, recent data from the Scottish Cancer Registry indicate that from 1988 to 2006, the highest incidence rates were seen for oropharyngeal cancer than for any other cancer types [132]. These changing epidemiological trends have been attributed to infection with high-risk HPV [132]. Case selection for previous studies investigating high-risk HPV related HNSCC in the Scottish population has been limited to oropharyngeal tumours [131], [321] and a mix of laryngeal and oropharyngeal tumours [4]. Whereas oropharyngeal cancers have also been the focal point of other cohort studies around the UK, this study is a retrospective analysis of a relatively heterogeneous group of patients who were diagnosed and treated in a single centre, the Ninewells Hospital (NHS Tayside). The availability of a head and neck database for case selection and the ease of sample acquisition made NHS Tayside an ideal choice for this study. Initially, 436 cases were selected from an existing database of head and neck cancer patients by employing known p16 status as an inclusion criterion. Following an assessment of the suitability of tissue specimens for downstream experiments and the presence of SCC, several cases were excluded from the cohort. Although the total sample size was reduced during this meticulous selection process, it ensured a robust cohort of 293 patients and is a larger sample size than those used in previous studies.

In the UK, crude incidence rates of head and neck cancer by gender show that there are 16 new cases for every 100,000 males and 8 for every 100,000 females - giving a male: female ratio of 2:1. Incidence rates are strongly related to age and, on an average, nearly 45% of cases diagnosed each year are in patients aged 65 years and over, although patterns may be different for males and females [34]. More than half of the patients in this study cohort were males and mean age of diagnosis was 64.5 years, which is in keeping with the general trends across the UK. At diagnosis, more than a third of the patient population were smokers and nearly a fourth had a history of heavy drinking. Alcohol consumption and tobacco use are major risk factors accounting for 75% of all head and neck cancers and their effects are synergistic when combined [50]. While smoking is strongly associated with laryngeal cancers, alcohol consumption tends to be linked to cancers of the oral cavity and pharynx [196].

In this cohort, cancers of the larynx and oral cavity constituted the majority of cases while OPSCC constituted around 21% of the patient population. These results are somewhat divergent from the WHO distribution wherein a majority of tumours in the upper aerodigestive tract arise in the oral cavity (43%) followed by the pharynx (31%) and the larynx (26%) [18]. It is likely that the primary tumour site distribution trends noted in this cohort is a reflection of smoking and alcohol consumption habits of patients that constitute it. However, recent reports suggest that an increasing number of patients with HNSCC do not present with a history of smoking and alcohol consumption at diagnosis. This trend is especially common in patients with HPV-positive OPSCC [44], [74].

Nearly half of the patient population (47%) in this cohort was diagnosed with late stage disease (Stage IVA & B). The stage of the disease at diagnosis will often determine prognosis and survival rate in patients with head and neck cancers and the best outcomes are seen in patients who are diagnosed early [143]. These findings may indicate a lack of awareness in the community on recognition of early signs and symptoms of head and neck cancer or a delay in seeking treatment or poor healthcare. Social deprivation has also been linked to HNSCC risk in high and low income countries across the world [51]. Indeed, higher cancer incidence and lower survival rates have also been reported in socioeconomically disadvantaged groups with deprivation gaps being the greatest for smoking related cancers [34], [234]. Individuals from socioeconomically deprived communities are often subjected to complex circumstances which demand more their time and attention and so, the risk of disease is often ignored [144]. Given that Dundee comprises 30% of the share of the most deprived communities in Scotland [271], the findings on negative health behaviours and disease presentation may indicate a need for patient education for preventive healthcare and screening programmes that are accessible to all communities.
Additionally, patients presenting with clinically advanced disease undergo multimodal treatment in the form of surgery, chemotherapy and/or radiotherapy which is associated with high morbidity [197], [228]. Indeed, only 19% of the patients in the cohort were treated with surgery alone, mainly those with early stage diseases, while most others were treated with radiation and/or chemotherapy.

Although a 5-year follow up is not complete for the cohort, the majority of the patients

were followed up for 3+ years. At the time of censoring, half of the patient population had died - the majority of them because of head and neck cancer - 49% were alive at the end of the study, comparable with survival rates of 71% at 1 year and 42% at 5 years in the European population [107].

Tobacco smoking, anatomic site of primary tumour, involvement of neck nodes, presence of extracapsular spread and treatment modality were found to significantly influence overall and cause specific survival. The detrimental effects of tobacco smoking on prognosis of head and neck cancer patients undergoing radiation therapy has been previously documented [44]. It has been hypothesised that decreased oxygenation and/or reduced effects of radiation induced killing of tumour cells may potentially compromise outcomes in these patients [112]. The 2-year OS and DSS were better for patients with laryngeal and oropharyngeal tumours and are similar to those reported by DAHNO for head and neck cancer patients in England [200]. The 2-year OS was reduced by half in patients with extracapsular spread, a finding that is consistent with data from a meta-analysis by Dunne *et al*. which showed a 5-year survival rate of 17% - 55.8% for neck metastasis with extracapsular spread and 44.6% - 76% neck metastasis without extracapsular spread [69].

Treatment modality is often determined by the disease stage at the time of diagnosis. Early stage disease or non-metastatic disease is usually treated with single modality therapy such as surgery or radiotherapy while patients with advanced disease undergo surgery with postoperative radiotherapy or by definitive radiotherapy followed by surgery, if necessary [164]. The vast majority of patients in this cohort were diagnosed with advanced head and neck cancer and nearly a quarter of the study population had undergone definitive radiotherapy. Findings on OS and DSS indicate that patients who had undergone definitive radiotherapy had the worst prognosis compared to those who had undergone radiotherapy in combination with surgery or chemotherapy or both. Indeed, overall and cause specific survival has been shown to be significantly improved with concomitant chemotherapy and radiation compared with definitive radiotherapy for advanced disease head and neck cancer [19], [176].

3.7 Summary

Clinical data and archival biopsy specimens were collected for a large heterogeneous

cohort of HNSCC from Tayside. Social demographics and clinical characteristics of the study population were retrospectively evaluated. The significant impact of smoking and disease stage on overall and disease specific survival highlights the need to address health behaviours and the lack of public awareness towards head and neck cancer in this population.

Chapter 4

PREVALENCE OF HUMAN PAPILLOMAVIRUS-RELATED HNSCC IN A TAYSIDE COHORT

The incidence of HPV-related HNSCC in the Western countries has risen rapidly over the last decade and is estimated to increase further in the next 20 years [246]. In the UK, 50 - 55% of OPSCCs have been attributed to infection with high risk HPV [132], [75]. On average, 75 - 80 patients are diagnosed and treated for head and neck cancer every year at the Ninewells Hospital which is the regional referral centre for patients in Tayside. One of the objectives of this study was to determine the prevalence of HPV-related HNSCC in the Tayside population by analysing archival tumour tissue (See Chapter 1, Section 1.7.5). This chapter will focus on the prevalence of high-risk HPV-related HNSCC in the sample population by retrospective analysis of archival FFPE specimens of patients diagnosed and treated between 2006 and 2011.

4.1 DNA extraction of FFPE archival tissue

4.1.1 Determining optimal thickness of tissue sections and comparing DNA extraction protocols

Formalin fixation and paraffin embedding (FFPE) is one of the most widely used archival storage and preservation methods in surgical diagnostic histopathology of disease tissue specimen. FFPE of tissue specimens preserves their morphology and cellular architecture and the tissues can be stored for extended periods of time with minimal maintenance. Furthermore, the ability to isolate nucleic acids and proteins from archived FFPE tissue blocks makes them a valuable resource for translational and molecular studies [140]**.** Isolation of DNA from archival FFPE tissue for molecular analyses is a multistep process involving many parameters that will ultimately define the quality and quantity of extracted DNA. Despite advances in biomolecular techniques, DNA extraction from FFPE tissues remains challenging.

The Tayside Tissue Bank, which serves as an extensive biorepository of tissue specimens, was a vital resource for obtaining FFPE blocks for this study. Considering the age of the tissue blocks and variability in specimen size, determining the amount of tissue and extraction protocol for optimal DNA quality and quantity was necessary. Three test FFPE tissue blocks of 0.1 cm^2, 0.5 cm^2 and 1 cm^2 were selected. The process of optimisation involved obtaining paraffin curls of 25 µm, 30 µm and 50 µm thickness

from each specimen. Tissue sections were deparaffinised and DNA extracted using protocols A (QIAamp DNA FFPE Tissue Kit) and B (DNA Lysis Buffer LC) as detailed in Section 2.4 of Chapter 2.

The most common method to determine DNA yield and purity is the measurement of ratios of absorbance at 260 nm and 280 nm (A260/280) and 260 nm and 230 nm (A260/230). As a general guideline, A260/280 ratio of 1.8 - 2.0 and A260/230 ratio of 2.0 - 2.2 are regarded as acceptable. DNA yield and absorbance ratios obtained with two different extraction methods were measured for each test specimen at different section thicknesses. The absorbance ratio of A260/280 for the smallest test specimen, Sample1, was found to be between 2 - 2.2 with Protocol A and 1.7 - 1.8 with Protocol B (Table 4.1). The A260/230 absorbance ratios were lower than the recommended value across all tissue thicknesses with both protocols. The total DNA yield in a volume of 50 µl was found to be appreciably better with extraction Protocol B.

Variable	Approximate area 0.1cm^2 (Sample 1)					
	QIAamp DNA FFPE Tissue Kit (A)			DNA Lysis Buffer LC (B)		
	5x5µm sections	3x10µm sections	5x10µm sections	5x5µm sections	3x10µm sections	5x10µm sections
DNA Concentration	16.5 ng/µl	7 ng/µl	18.5 ng/µl	29 ng/µl	44 ng/µl	53.5 ng/µl
$A_{260/280}$	2.2	2	2.2	1.7	1.7	1.8
$A_{260/230}$	0.2	-11.7	0.2	0.8	1	1.1
Yield in 50 µl volume	0.8 µg	0.4 µg	0.9 µg	1.5 µg	2.2 µg	2.5 µg

Table 4.1: Comparison of DNA extraction Protocols A and B for DNA purity by Nanodrop assessment for Sample 1. The absorbance ratio of A260/280 for the smallest test specimen, was found to be between 2 - 2.2 with Protocol A and 1.7 - 1.8 with Protocol B. The A260/230 absorbance ratios were variable and lower than the recommended value across all tissue thicknesses. The total DNA yield in a volume of 50 µl was found to be appreciably better with extraction Protocol B.

For the second test specimen (Sample 2), an A260/280 absorbance ratio of 2.0 with Protocol A and 1.8 with protocol B was noted. These values were also consistent across all tissue thicknesses while those of A260/230 absorbance ratio were variable. However, DNA yield was considerably higher with Protocol B (Table 4.2).

Variable	Approximate area 0.5 cm^2 (Sample 2)					
	QIAamp DNA FFPE Tissue Kit (A)			DNA Lysis Buffer LC (B)		
	5x5µm sections	3x10µm sections	5x10µm sections	5x5µm sections	3x10µm sections	5x10µm sections
DNA Concentration	165 ng/µl	105 ng/µl	155 ng/µl	273.5 ng/µl	104 ng/µl	408 ng/µl
$A_{260/280}$	2	2	2	1.8	1.8	1.8
$A_{260/230}$	2.5	2.1	1	1.5	1.8	1.4
Yield in 50 µl volume	8.3 µg	5.3 µg	7.8 µg	13.7 µg	5.2 µg	20.4 µg

Table 4.2: Comparison of DNA extraction Protocols A and B for DNA purity by Nanodrop assessment of Sample 2. A consistent A260/280 absorbance ratio of 2.0 with Protocol A and 1.8 with protocol B was noted while those of A260/230 absorbance ratio were variable. DNA yield was considerably higher with Protocol B.

A similar pattern of absorbance ratios was observed following extraction of DNA from the third and largest tissue sample with DNA yield being higher for Protocol B (Table 4.3).

Variable	Approximate area 1 cm^2 (Sample 3)					
	QIAamp DNA FFPE Tissue Kit (A)			DNA Lysis Buffer LC (B)		
	5x5µm sections	3x10µm sections	5x10µm sections	5x5µm sections	3x10µm sections	5x10µm sections
DNA Concentration	44 ng/µl	26.5 ng/µl	31.5 ng/µl	82 ng/µl	33 ng/µl	114 ng/µl
$A_{260/280}$	2	1.9	2	1.7	1.8	1.8
$A_{260/230}$	0.4	3	0.3	0.7	1.7	0.7
Yield in 50 µl volume	2.2 µg	1.3 µg	1.6 µg	4.1 µg	1.7 µg	5.7 µg

Table 4.3 Comparison of DNA extraction Protocols A and B for DNA purity by Nanodrop assessment of Sample 3. An A260/280 absorbance ratio of 1.9 - 2.0 with Protocol A and 1.7 - 1.8 with protocol B was noted across all tissue thicknesses while those of A260/230 absorbance ratio were variable. However, DNA yield was considerably higher with Protocol B.

The primary objective of comparing tissue thickness and extraction protocols was to determine the best approach for obtaining amplifiable and pure DNA for genotyping. Accordingly, PCR amplification of the human β-globin gene from template DNA (100

ng) obtained with extraction protocols A and B from different tissue thicknesses of each test specimen was compared. The PCR products were visualised by agarose gel electrophoresis to assess DNA quality and quantity.

For Sample 1, faint bands corresponding to the amplified β-globin gene (260bp) were visible for DNA obtained with both extraction protocols and different tissue thicknesses (Figure 4.1(A)). However, the strongest PCR product was obtained with Protocol A from a tissue thickness of 50 µm. PCR amplification of the β-globin gene for Sample 2 only yielded products with template DNA obtained with Protocol A at 25 and 50 µm tissue thickness (Figure 4.1(B)). For Sample 3, a strong PCR product was observed with template DNA obtained with Protocol A at a tissue thickness of 50 µm (Figure 4.1(C).

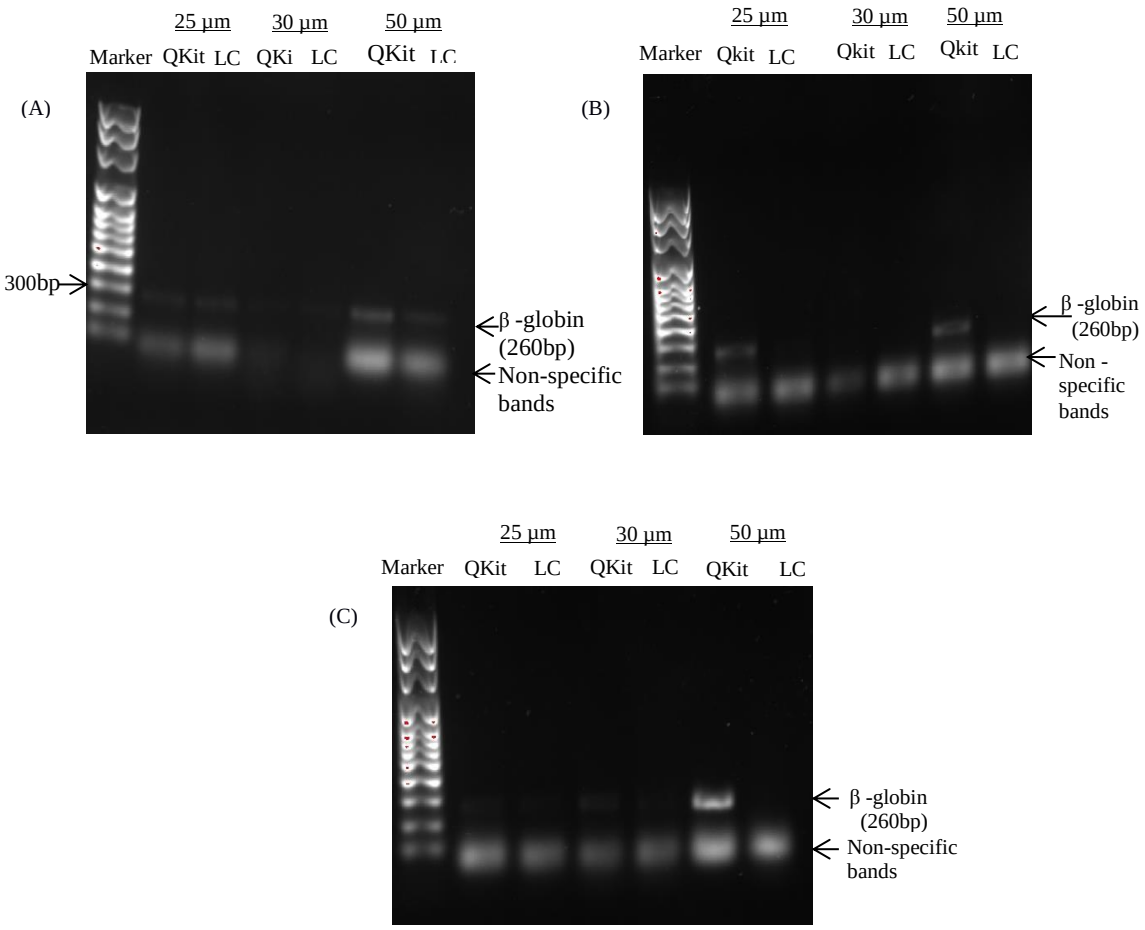

Figure 4.1 Comparison of DNA extraction Protocols A and B by PCR amplification of the Human β-globin gene in test samples 1 (A), 2 (B), and 3 (C). Protocols A (QIAamp DNA FFPE Kit) and B (DNA lysis buffer LC) were employed in extracting DNA from test FFPE tissue specimens of varying sizes 1 (0.1 cm^2), 2 (0.5 cm^2) and 3 (1 cm^2) at tissue thicknesses of 25, 30 and 50 µm each. Extracted DNA was assessed for quality by amplification of a 260bp fragment of the human β-globin gene. Bands corresponding to template DNA with Protocol A at a tissue thickness of 50 µm were consistently strong and clear. The additional bands noted in figures (A), (B) and (C) were identified as non-specific bands (discussed in Section 4.2.5).

Assessment of the purity of DNA using a Nanodrop showed no significant distinction among the three tissue thicknesses, however, the DNA yield was consistently higher with Protocol B. On the contrary, PCR analysis of template DNA demonstrated that extraction Protocol A provided the best results with consistently strong amplification of DNA from a tissue thickness of 50 µm. Subsequent DNA extractions for archived study FFPE tissue specimens were therefore carried out using the QIAamp DNA FFPE tissue kit (Protocol A).

4.1.2 Extraction of genomic DNA from study specimens for PCR analysis

Archived FFPE tissue blocks were obtained from the Tayside Tissue Bank and tissue specimens were assessed for their suitability in PCR analysis. Genomic DNA was extracted from 320 specimens using the QIAamp FFPE Tissue kit. The concentration and absorbance ratios of extracted DNA samples showed that the absorbance ratio of A260/280 was 1.9 - 2.1 for most samples while that of A260/230 was 2 - 2.3. Depending on the size of the specimen, DNA concentration was variable across samples with concentrations as low as 4 ng/µl for a few specimens.

4.2 Detection of HPV DNA in the archived specimens

4.2.1 Optimising PCR reactions

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for the amplification of DNA segments. Successful amplification of a DNA sequence is dependent on several parameters which include template DNA, primers, DNA polymerase, reagents such as dNTPs and magnesium and thermal cycling temperatures. Optimisation of PCR reagent concentrations and cycling conditions improves product yield and reproducibility between reactions, while reducing non-specific and unwanted products.

PCR analysis for this study was performed using the Taq DNA Polymerase system supplied by New England Biolabs, which provides an optimised protocol and a buffer solution with added dNTPs and magnesium. Ideal thermal cycling temperatures were subsequently ascertained for different primer pairs used in the study.

4.2.2 Selecting controls for PCR Reactions

The use of negative and positive controls is important to validate the results of gene expression obtained by PCR. DNA extracted from HaCaT, SiHa or HeLa cell lines were employed as positive controls for the detection of human β-globin, HPV L1 and HPV E1 respectively. Figure 4.2 below is an illustration of the quality of template positive control DNA obtained from cell lines.

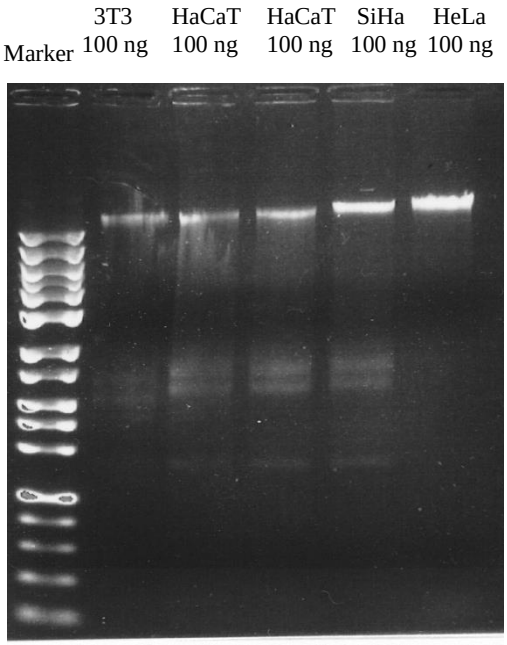

Figure 4.2: Agarose gel electrophoresis image of template DNA to assess quality of DNA extracted from HaCaT, SiHa and HeLa cell lines. DNA extracts from HaCaT, SiHa and Hela cell lines were employed as controls for PCR amplifications of the human β-globin gene, HPV L1 and E1 genes respectively. SiHa and HeLa cell lines harbour HPV-16 and HPV-18 respectively and were employed as controls for PCR amplification of HPV-16 E7 and HPV-18 E7 genes.

4.2.3 Selecting HPV types for investigation

A major portion of evidence on the presence of HPV in HNSCC in the literature points to high-risk HPV-16 as an important causative factor [195]. In order to ascertain the incidence of other high risk HPV types prevalent in the sample patient population from the Tayside catchment area, investigations for this study used, as a guideline, the most common HPV types which had been identified by various scientific groups in British patient populations. As such, the prevalence of HPV types 16, 18, 33, and 52 was investigated [4], [131], [321], [264], [178], [75].

4.2.4 Determining optimal cycling conditions for each PCR

Target genes human β-globin, HPV L1 and E1 were amplified with consensus primer pairs GH20/PC04, MY09/MY011 and CP1/CPIIG respectively. E7 genes of HPV types 16, 18, 33 and 52 were amplified with type specific primer pairs as outlined in Chapter 2, Table 2.2.

A guideline annealing temperature for each primer pair was obtained from relevant literature. The protocol recommended by New England Biolabs successfully amplified target genes for most primers albeit with weak and non-specific products in certain instances. Therefore, parallel reactions were set up with different annealing temperatures to optimise the PCR product. Figure 4.3 below is a representation of amplification of target gene HPV E1 at various annealing temperatures, of which 52^{o}C was found to be the most ideal.

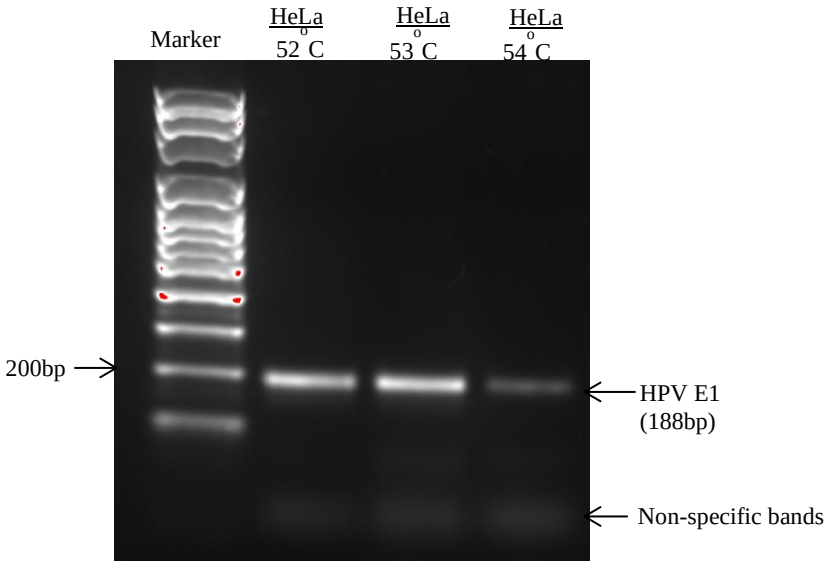

Figure 4.3: Optimising consensus primers. Parallel PCR reactions were set up to determine the optimal annealing temperature for each primer pair. Template DNA from HeLa cell extracts was employed to amplify a188bp fragment of the HPV E1 gene using CPI/IIG primers. An annealing temperature of 52^{o}C was found to be the most ideal for this primer pair.

Test reactions were also carried out to ensure that each primer pair was specific to the target gene of interest. Figures 4.4 (A) and (B) are an illustration of the specificity of primer pairs amplifying the HPV-16 E7 and HPV-18 E7 genes.

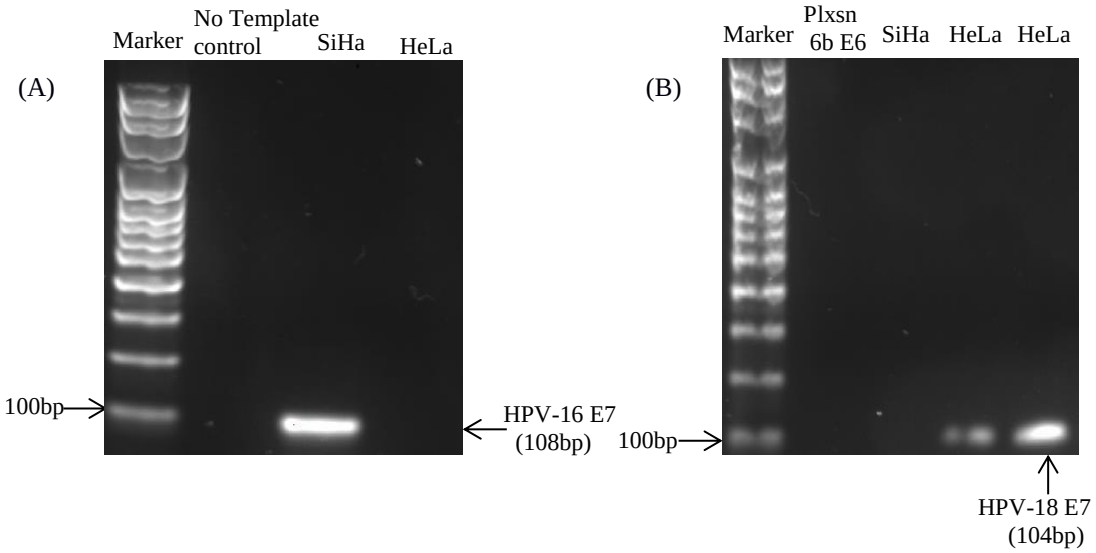

Figure 4.4 (A) & (B): Optimising type specific primers (A) A PCR reaction was carried out to amplify a 108bp fragment of the HPV-16 E7 employing a type specific primer pair. DNA extracted from SiHa and HeLa cell lines which harbour HPV-16 and 18 respectively, were used as template DNA. As expected, a PCR product of the correct size is seen corresponding to the sample containing the SiHa extract. (B) A similar PCR reaction was carried out to amplify the HPV-18 E7 gene. A PCR product of correct molecular weight is seen corresponding to the samples containing HeLa. The plasmid Plxsn 6b E6 was employed as a negative control along with DNA from SiHa cell lines.

4.2.5 Troubleshooting non-specific bands

While all the PCRs successfully amplified target genes, the appearance of non-specific bands prompted further investigation. When the experiments were repeated with variations in primer/template concentrations and cycling conditions**,** the additional bands persisted (Figure 4.5A). However, when a reaction was carried out with single primer controls (Figure 4.5 B) non-specific bands corresponding to these primers were not seen. The non-specific PCR products were therefore attributed to primer dimerisation. Further attempts to eliminate these bands were deemed unnecessary as they did not interfere with any of the target sequences.

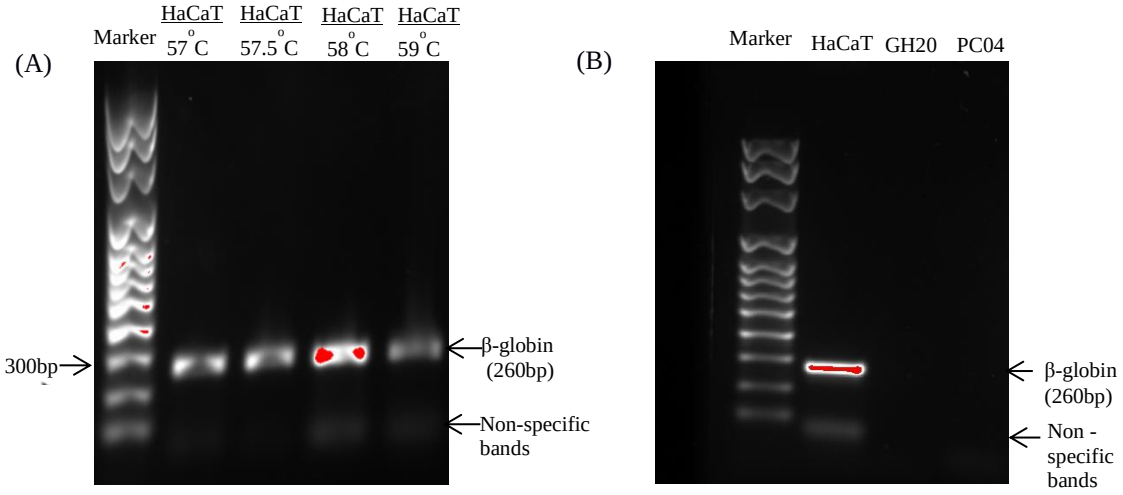

Figure 4.5 (A) & (B): Troubleshooting non-specific PCR products (A) Parallel PCR reactions at annealing temperatures ranging from 57°C – 59°C were set up to amplify human β-globin from DNA extracted from HaCaT cell lines. Increasing annealing temperature failed to completely eliminate the non-specific bands. (B) In order to investigate the possibility of primer dimerisation a PCR reaction was carried out to amplify human β-globin using HaCaT template DNA and single primers from the GH20/PC04 pair as controls. A non-specific product was only visible in Lane 2 corresponding to the template HaCaT DNA suggesting that the non-specific bands may correspond to primer dimers.

4.2.6 Determining the presence of amplifiable DNA with human β-globin gene

The quality of DNA extracted from FFPE specimens can vary from one sample to another depending on factors such as type of fixative, duration of fixation and post fixation storage [120]. Amplifying a fragment of a housekeeping gene is an effective way to assess the quality of DNA extracted from archived FFPE tissue specimens.

Extracted DNA from 320 FFPE specimens was subjected to PCR to amplify a 260bp fragment of the human β-globin gene using the GH20/PC04 primer pair with DNA from HaCaT and SiHa cell lines serving as positive controls. Out of 320 samples, 293 (92%) were positive for β-globin. PCR products visualised as bands on the gel were of varying intensities ranging from very strong bands to weak bands. Figure 4.6 below shows β-globin amplification in 24 patient DNA samples. In the detailed image of Gel 1, bands corresponding to the β-globin gene are visible for Specimen IDs 66-71 along with positive controls HaCaT and SiHa. No band is visible in relation to the 'No Template Control'.

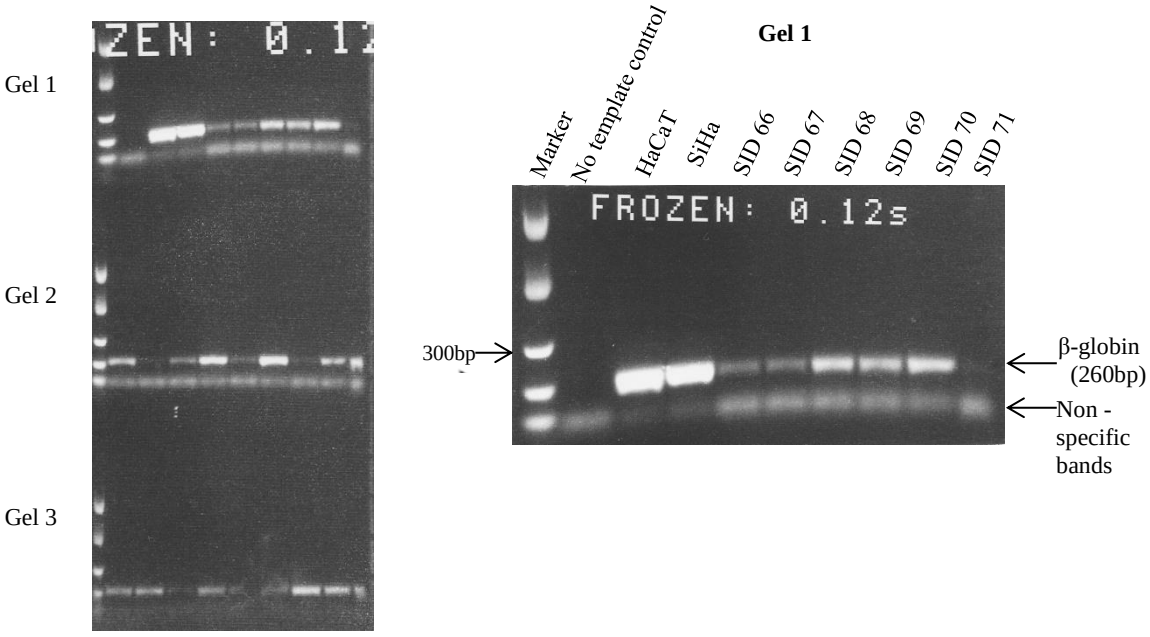

Figure 4.6: PCR assessment of DNA quality. DNA extracted from archived FFPE specimens was analysed for the presence of human β-globin by amplifying a 260bp fragment of the β-globin gene using the GP04/PC05 primers. Image of Gel 1 has been enlarged to show β-globin was amplification in samples 66-71. DNA extracted from SiHa and HaCaT cell lines was used as positive controls.

4.2.7 Determination of the presence of HPV L1 and E1 genes

The 27 extracted DNA samples negative for human β-globin gene were excluded from the cohort. The remaining 293 samples were put through PCR to amplify a 450bp fragment of the HPV consensus L1 gene using the MY09/MY011 primer pair. Of the 293 samples, 42 were positive for HPV L1 gene. Samples negative for HPV L1 gene were further investigated for the presence of consensus HPV E1 gene by performing PCR to amplify a 188bp fragment of the E1 gene using CPI/CPIIG primers. DNA from HeLa or SiHa cell lines served as positive controls for both the sets of PCR reactions. Eleven samples were found to be positive for HPV E1. Figure 4.7 below is a representation of PCR amplification of HPV E1 gene from Specimens 139 - 150.

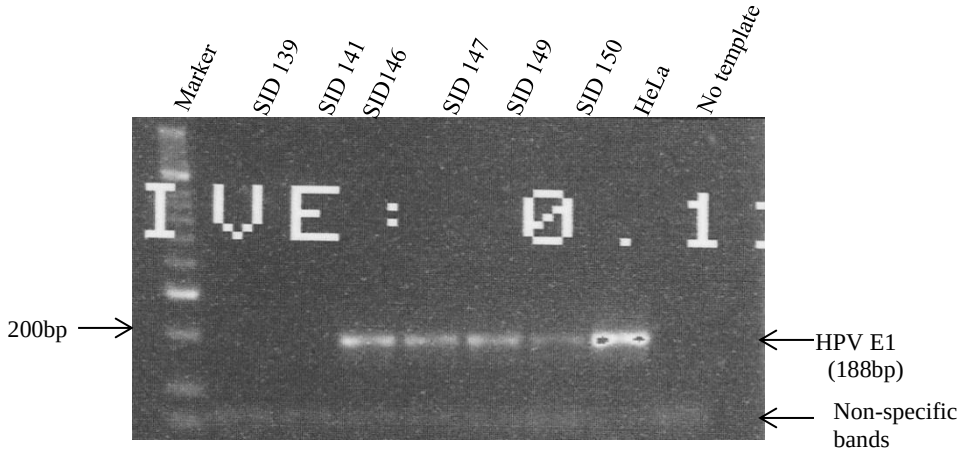

Figure 4.7: PCR analysis for the HPV E1 gene. DNA extracted from archived FFPE specimens was analysed for the presence of HPV E1 by amplifying a 188bp fragment of the HPV E1 gene using the consensus CPI/IIG primers. 'SID' denotes Sample ID. DNA extracted from HeLa cell lines was used as a positive control, and SIDs 146 -150 were found to be positive for the E1 gene.

4.2.8 Determining types of high-risk HPV

The 53 samples positive for HPV L1 or E1 were investigated further to determine the specific HPV genotype by amplifying fragments of the E7 gene of HPV types 16, 18, 33 and 52. Fifty samples positive for HPV L1 or E1 were also positive for HPV-16 E7, two samples were positive for HPV-18 E7 and one for HPV-33 E7. Six of the genotyped samples were sequenced to confirm the specificity of the PCR products. Figure 4.8 is an illustration of PCR amplification of the HPV-16 E7 gene in specimens 2, 14, 45, 55, 84, 88, 133, 134 and 348.

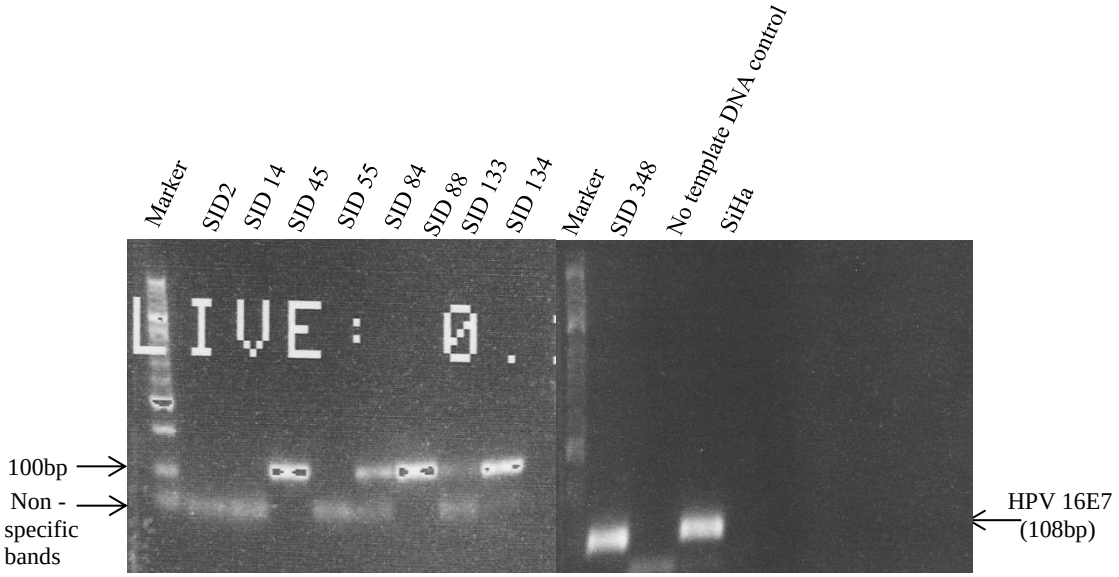

Figure 4.8: PCR analysis for HR-HPV type 16. DNA extracted from archived FFPE specimens was analysed for the presence of HPV 16 by amplifying a 108bp fragment of HPV 16 E7 gene using type specific primers. 'SID' denotes Sample ID. DNA extracted from SiHa cell lines was used as a positive control (image of another gel with controls from the same experiment). As evident in this gel, SIDs 45, 84, 88, 134 and 348 were found to be positive for HPV 16.

The workflow diagram below summarises the overall final results of the PCR component of the study (Figure 4.9).

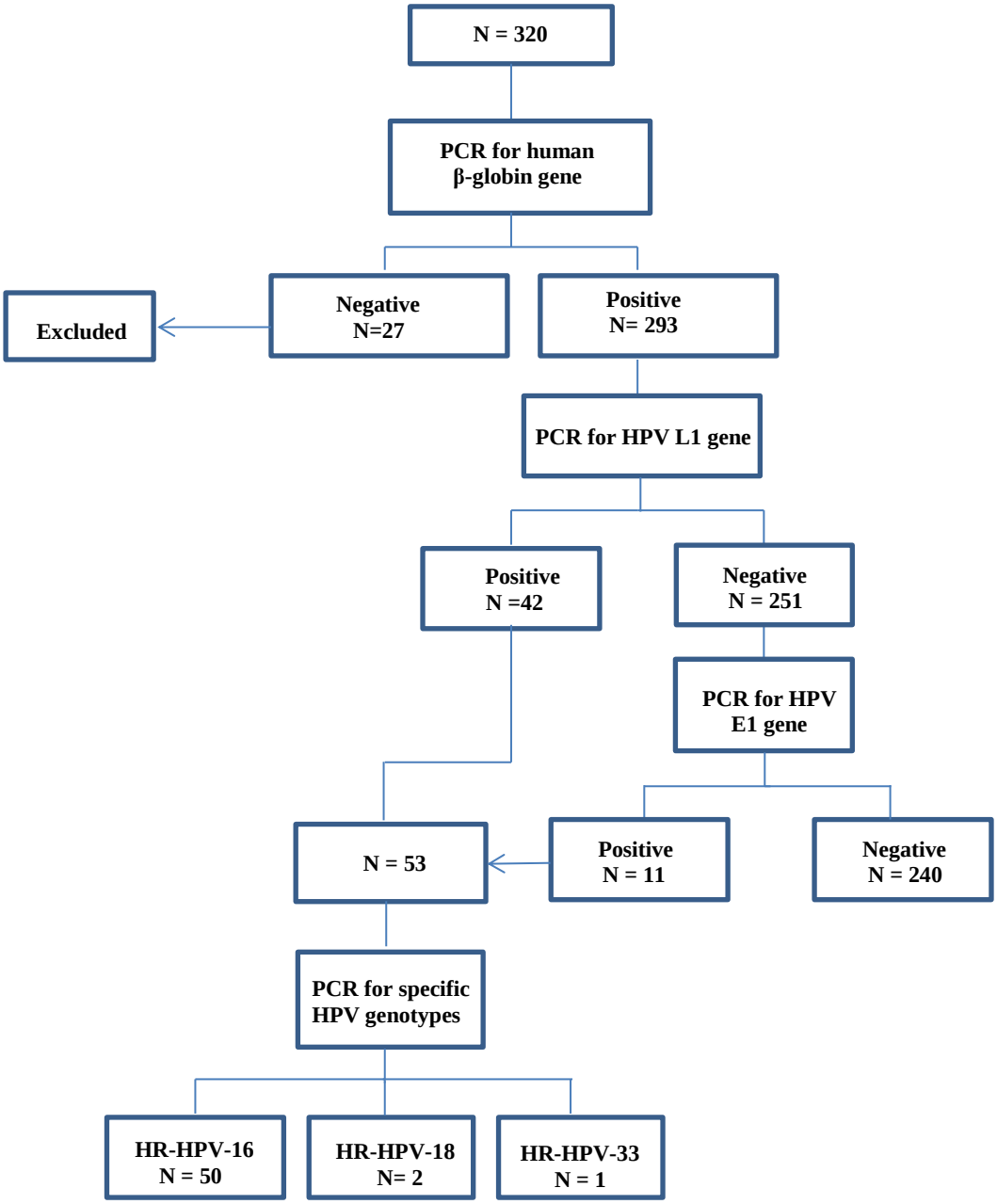

Figure 4.9: PCR workflow diagram. Study samples were initially tested for DNA quality by amplifying the house keeping gene human β-globin. Samples negative for β-globin were excluded from the study. The remaining samples (n=293) were genotyped for HR-HPV using consensus primers MY09/MY011 and CPI/IIG followed by type specific primers for HPV types 16, 18, 33 and 52. HR-HPV type 16 was detected in 50 samples, HR-HPV type 18 in 2 samples and type 33 in 1 sample.

4.3 Prevalence of HPV in the study population

4.3.1 p16 status of the study cohort

A known p16 status for each patient was a key inclusion criterion for this study. Archived biopsy or resection specimens of patients were selected from an existing database of HNSCC carcinoma patients based on the availability of a p16 status. Out of 293 patients, 47 (16%) were positive for p16 and the remaining 246 (84%) were p16-negative (Figure 4.10).

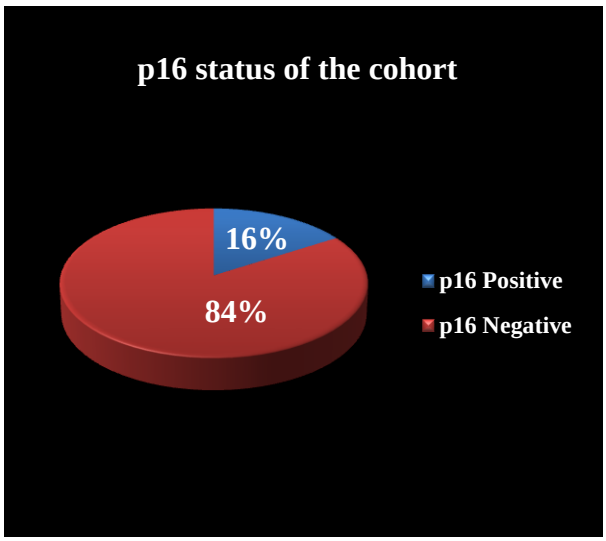

Figure 4.10: p16 status of the study cohort. Archived FFPE tumour samples were selected for the study based on the availability of a p16 status for each patient. Out of 293 patients, 47 (16%) were p16-positive and the remaining 246 (84%) were p16- negative.

4.3.2 Variability in patient demographics and tumour histopathology by p16 status

The p16-positive and negative groups were compared for significant associations based on demographics and tumour histopathology (Table 4.4). More than half of the patients in the p16-positive group were under the age of 58 years ($P = 0.002$). They were either non-smokers or ex-smokers while patients in the p16-negative group were predominantly current smokers ($P < 0.001$). More than half of the patients in the p16-positive group (28/47; 60%) were also light to moderate drinkers ($P < 0.05$).

Significant differences were noted between the two patient groups with respect to anatomic site of primary tumour, grade of differentiation and disease stage (Table 4.4). The majority of p16-positive tumours (60%) arose in the oropharynx and were either poorly differentiated (23%) or moderate to poorly differentiated (25%), while p16-

negative tumours were more common in the larynx (99%) or oral cavity (95%) and were likely to be either well or moderately differentiated ($P < 0.001$, $P = 0.002$). Although p16-positive patients were more likely to present with late stage disease ($P = 0.003$), overall survival was better compared to the negative group ($P = 0.004$**)** and had a decreased likelihood of recurrence as well ($P = 0.03$).

Variable	p16-negative (n=246)	p16-positive (n=47)	Total No. (n=293)	Pearson's CHI Square
Age				
≤ 58	67 (73%)	25(27%)	92	
59 – 64	51(84%)	10 (16%)	61	
65 – 73	65 (90%)	7 (10%)	72	***P* = 0.002**
≥ 74	63 (93%)	5 (7%)	68	
Total	246	47	293	
History of smoking				
Non smoker	31 (66%)	16 (34%)	47	
Smoker [d]	116 (93%)	8 (7%)	124	
Ex-smoker	59 (80%)	15 (20%)	74	***P* < 0.001**
Total	206	39	245	
History of alcohol consumption				
Light-moderate drinker	89 (76%)	28 (24%)	117	
Heavy drinker	63 (89%)	8 (11%)	71	
Non drinker	32 (94%)	2 (6%)	34	***P* = 0.03**
Ex-heavy drinker	9 (100%)	0 (0%)	9	
Alcohol status unknown[a]	53 (85%)	9 (15%)	62	
Total	246	47	293	
Site of primary tumour				
Oral cavity	94 (95%)	5 (5%)	99	
Oropharynx	24 (40%)	36 (60%)	60	
Larynx	87 (99%)	1 (1%)	88	***P* < 0.001**
Other[b]	41 (89%)	5 (11%)	46	
Total	246	47	293	

Grade of tumour differentiation				
Well differentiated	5 (100%)	0 (0%)	5	
Moderately differentiated	111 (93%)	8 (7%)	119	***P* = 0.002**
Poorly differentiated	88 (77%)	27 (23%)	115	
Moderate to poorly differentiated	21 (75%)	7 (25%)	28	
Total	225	42	267[a]	
Disease stage				
Stage I	46 (98%)	1 (2%)	47	
Stage II	39 (93%)	3 (7%)	42	
Stage III	36 (84%)	7 (16%)	43	***P* = 0.003**
Stage IVA & IV B	105 (76%)	33 (24%)	138	
Unknown[c]	8 (89%)	1 (11%)	9	
Total	234	45	279	
Overall survival				
Alive	112 (78%)	32 (22%)	144	
Dead	134 (90%)	15 (10%)	149	***P* = 0.004**
Total	246	47	293	
Recurrence				
Yes	51 (93%)	4 (7%)	55	
No	195 (82%)	43 (18%)	238	***P* = 0.03**
Total	246	47	293	

a. Alcohol status unknown- data on alcohol history at diagnosis was unavailable
b. Other – includes hypopharynx, pharynx, nasopharynx and unknown primary
c. Unknown –where primary tumour was unknown and stage of disease could not be determined
d. The categories 'Light smoker', 'Moderate smoker', 'Heavy smoker' and 'other' introduced in Table 3.2 were collapsed to into the category 'smoker'

Table 4.4: Baseline characteristics of patients stratified based on p16 status. Significant differences were noted between patients with p16-positive and negative tumours in social demographics, site of primary tumour, histological grade of tumour differentiation, disease stage, overall and recurrence free survival.

4.3.3 Relationship between p16 status and survival

After a median follow-up period of 46 months, the p16-positive group had a larger proportion of survivors (32/47; 68%) compared to the p16-negative group (112/246; 45%). A Kaplan Meier analysis of overall survival rates demonstrated favourable prognosis for patients with p16-positive tumours (Figure 4.11). Mean survival time (in

months) for p16-positive patients was 67 months (95% Confidence Interval 57 – 77) compared to only 55 months for p16-negative patients (95% Confidence Interval 49 – 62) (Log Rank (Mantel-Cox) CHI Sq. 6.4 (df 1) *P* = 0.01).

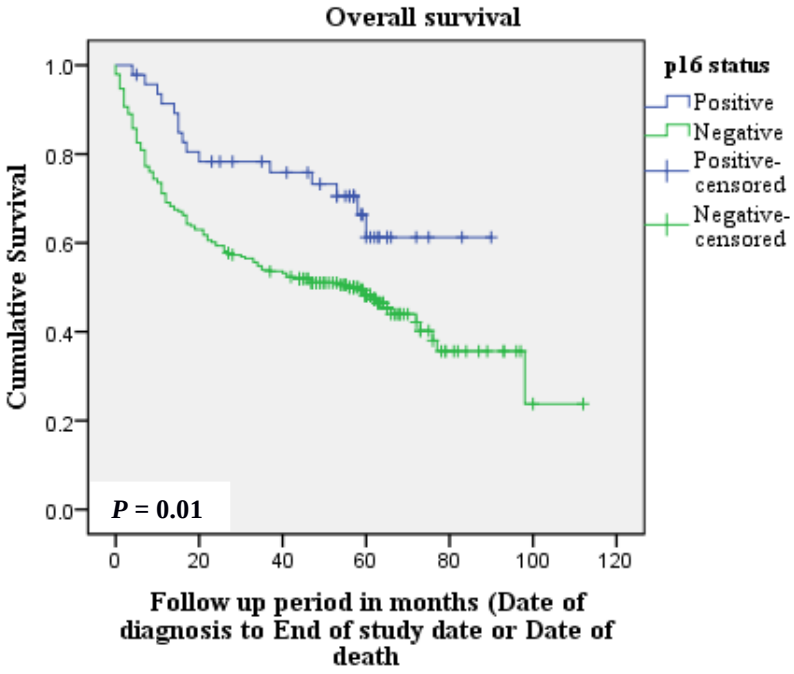

Figure 4.11: Kaplan-Meier plot for overall survival based on p16 status. Overall survival for patients with p16-positive tumours (represented by the blue line) was significantly better compared to patients with p16-negative tumours (represented by the green line).

The median follow-up period for recurrence free survival was 43 months and a greater incidence of recurrence was recorded in p16-negative compared to those with p16-positive tumours. A Kaplan Meier analysis demonstrated improved disease free survival for p16- positive patients compared to their negative counterparts (Figure 4.12). Mean disease free survival time for p16-positive patients was 75 months (95% Confidence Interval 68.3 – 81.5) compared to 64 months for p16-negative patients (95% Confidence Interval 63.5 – 74.3) (Log Rank (Mantel –Cox) CHI Sq. 5.571 (df 1) *P* = 0.018).

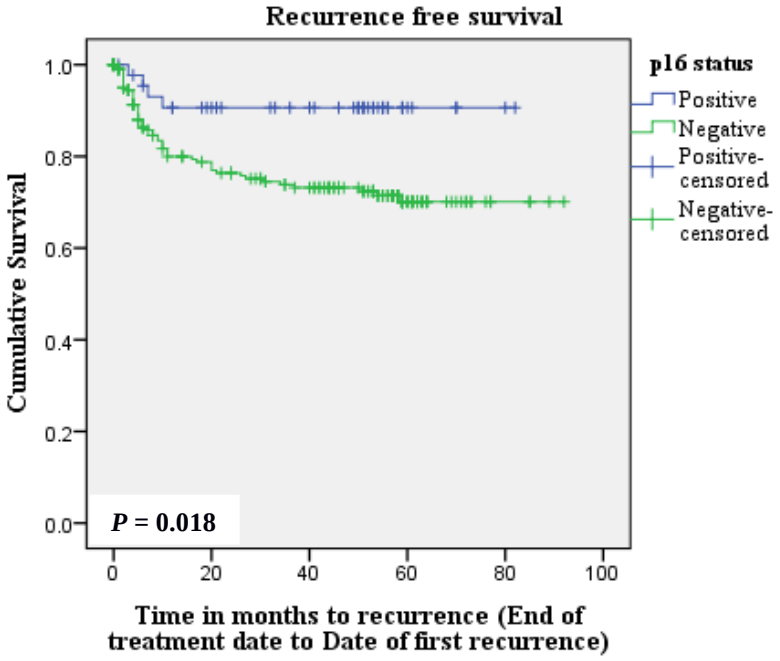

Figure 4.12: Kaplan-Meier plot for recurrence free survival based on p16 status. Patients with p16-positive tumours (n=47) represented by the blue line demonstrated better disease free survival compared to those with p16-negative tumours (n=246) represented by the green line.

A Kaplan Meier analysis was performed to determine disease specific survival in p16-positive patients and negative patients (Figure 4.13). Mean disease specific survival time for p16-positive patients was 79 months (95% Confidence Interval 71.6 – 87.2) compared to 83 months for p16 negative patients (95% Confidence Interval 74.8 – 90.6). No significant improvement in disease specific survival was noted between the two patient groups (Log Rank (Mantel –Cox) CHI Sq. 2.39 (df 1) $P = 0.122$).

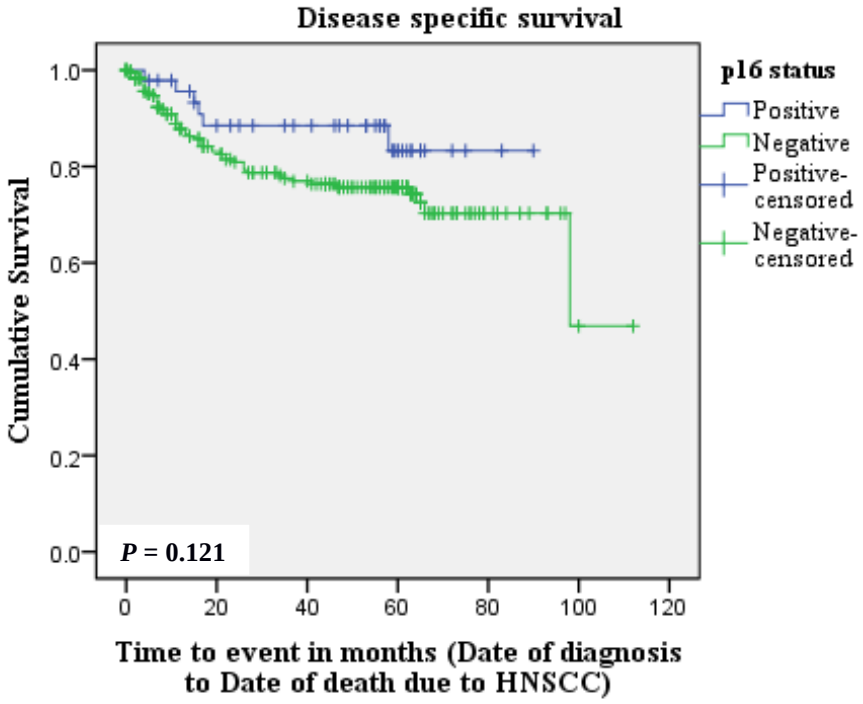

Figure 4.13: Kaplan-Meier plot for disease specific survival based on p16 status. Although patients with p16-positive tumours (represented by the blue line) had better disease specific survival compared to those with p16-negative tumours (represented by the green line), it was not found to be statistically significant.

4.3.4 HPV prevalence based on detection of viral DNA

PCR amplification of viral DNA revealed that 18% (n=53) of the study population was HPV DNA positive. Of the positive samples, an overwhelming majority were high-risk HPV type 16 (94%), while types HPV types 18 and 33 constituted 6%. Figure 4.14 below summarises the prevalence of high-risk HPV DNA in the study population.

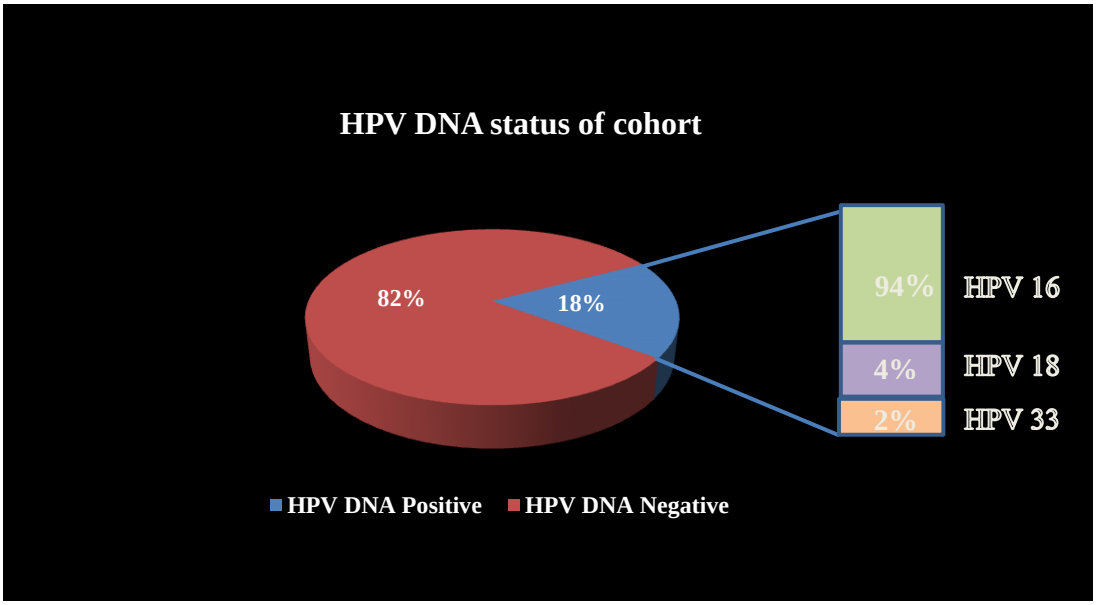

Figure 4.14: Prevalence of high-risk HPV DNA in the study cohort. Archival FFPE tissue specimens were genotyped by PCR for high-risk HPV. While the vast majority of patient samples were negative for viral DNA (n= 240), 50 samples were positive for high-risk HPV 16, 2 for HPV type 18 and 1 for HPV type 33.

4.3.5 Prevalence of HPV based on HPV DNA status and p16 overexpression

In order to ascertain the actual prevalence of HPV in the study population, patients were classified based on the presence of HPV DNA and/or p16 overexpression. Four groups were identified: HPV DNA-ve/p16-ve, HPV DNA-ve/p16+ve, HPV DNA+ve/p16-ve and HPV DNA+ve/p16+ve as described by Weinberger *et al*. [319]. Tumours were defined as 'true positive' only if they were positive for both HPV DNA and p16, and 'true negative' when they were negative for both. Tumours positive for either HPV DNA or p16 were defined as 'Equivocal' [75]. The bar chart below represents the proportion of the four groups in the study cohort (Figure 4.15). Almost 80% of the cohort was true negative and 14% true positive. Equivocal groups constituted the remaining 6% and were excluded from subsequent analyses.

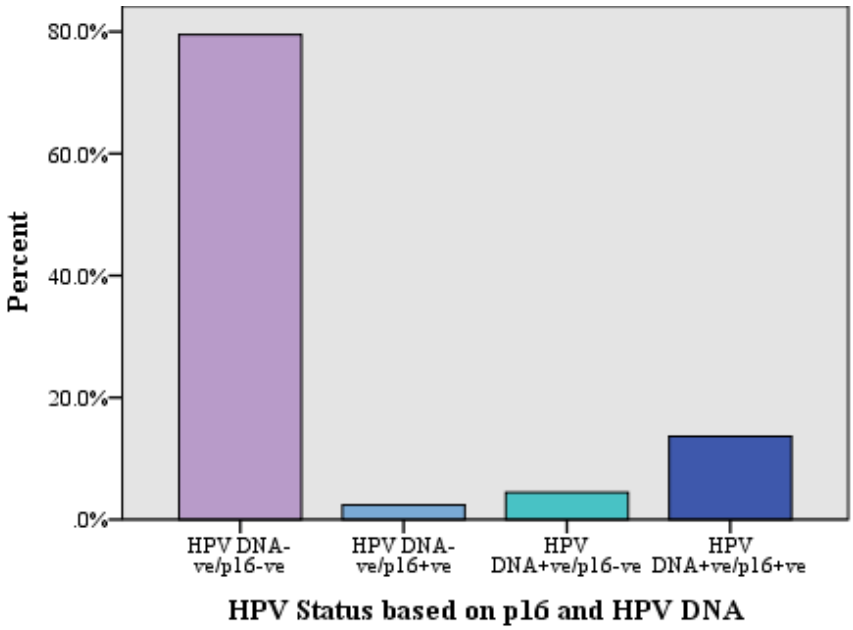

Figure 4.15: Classification of study cohort based on HPV DNA and/or p16 expression. Patients were divided into 4 groups - HPV DNA-ve/p16-ve or 'True negative' (n= 233), HPV DNA-ve/p16+ve or 'Equivocal' (n=7), HPV DNA+ve/p16-ve or 'Equivocal' (n=13), HPV DNA+ve/p16+ve or 'True positive' (n=40).

4.3.6 Variability in patient demographics and tumour histopathology by HPV status

The majority of HPV-positive patients were found to be in the younger age group of 58 years or under ($P = 0.001$) and were either 'non-smokers' or 'Ex-smokers', compared to the HPV-negative group where most patients were 'Current smokers' ($P < 0.001$) (Table 4.5). Patients with HPV-positive tumours were more likely to be 'light to moderate drinkers' at the time of diagnosis compared to those with HPV-negative tumours who were either 'light to moderate drinkers' or 'heavy drinkers' ($P = 0.005$). Surprisingly, the number of cases diagnosed did not increase with each year, although a surge in cases diagnosed was noted in the year 2010. HPV-positive tumours arose predominantly in the oropharynx (33/40; 83%) while HPV-negative tumours arose in the oral cavity (93/233; 39%) and the larynx (84/233; 36%). Further analysis of the anatomic site of origin of HPV-positive tumours revealed that 24 out of 40 tumours arose in the tonsils and 9 in the base of the tongue (Table 4.6). Although both groups presented with late stage disease, HPV-positive patients were more likely to present with nodal metastasis ($P < 0.001$). Substantial differences in histopathological features such as tumour differentiation, extracapsular spread and presence of tumour invasive front were noted between the groups. While a significant proportion of HPV-negative tumours were either well or moderately differentiated, HPV-positive patients were

likely to present with poor or moderate to poorly differentiated tumours ($P < 0.001$). HPV-positive tumours were also less likely to exhibit extracapsular spread ($P = 0.001$) and an invasive cohesive front ($P < 0.001$), both of which are features of tumour aggression. At the time of censoring, more than half of the patients from the HPV-negative group were deceased compared to only 30% (12/40) from the HPV-positive group ($P = 0.002$). No significant associations were found between histological features of tumours such a presence of lymphovascular and perineural invasion and tumour HPV status presumably because of the large amount of missing data for these variables

Variable	HPV-negative (n=233)	HPV- positive (n=40)	Total No. (n=273)	Pearson's CHI Square
Age				
≤ 58	60 (72%)	23 (28%)	83	
59 – 64	47 (87%)	7 (13%)	54	
65 – 73	64 (91%)	6 (9%)	70	***P* = 0.001**
≥ 74	62 (94%)	4 (6%)	66	
Total	233	40	273	
History of smoking				
Non smoker	27 (64%)	15 (36%)	42	
Current smoker	112 (97%)	4 (3%)	116	
Ex-smoker	56 (80%)	14 (20%)	70	***P* = 0.000**
Total	195	33	228	
History of alcohol consumption				
Light to moderate drinker	78 (76%)	25(24%)	103	
Heavy drinker	63 (91%)	6 (9%)	69	
Non drinker	32 (94%)	2 (6%)	34	***P* = 0.005**
Ex-heavy drinker	9 (100%)	0 (0%)	9	
Total	182	33	215	
Year of diagnosis				
2006	26 (90%)	3 (10%)	29	
2007	60 (85%)	11 (15%)	71	
2008	38 (100%)	0 (0%)	38	
2009	42 (89%)	5 (11%)	47	***P* = 0.005**
2010	32 (70%)	14 (30%)	46	
2011	35 (83%)	7 (17%)	42	
Total	233	40	273	

Site of primary tumour				
Oral cavity	91 (97%)	3 (3%)	94	
Oropharynx	21 (39%)	33 (61%)	54	
Larynx	84 (100%)	0 (0%)	84	***P* = 0.000**
Other[a]	37 (90%)	4 (10%)	41	
Total	233	40	273	
Grade of tumour differentiation				
Well differentiated	5 (100%)	0 (0%)	5	
Moderately differentiated	110 (96%)	5 (4%)	115	
Poorly differentiated	80 (77%)	24 (23%)	104	***P* = 0.000**
Moderate to poorly differentiated	19 (76%)	6 (24%)	25	
Total	214	35	249	
Disease staging				
Stage I	42 (98%)	1 (2%)	43	
Stage II	39 (95%)	2 (5%)	41	***P* ≥ 0.05 (NS)**
Stage III	34 (85%)	6 (15%)	40	
Stage IVA & IVB	101 (78%)	29 (22%)	130	
Unknown[b]	5 (83%)	1 (17%)	6	
Total	221	39	260	
Neck node status				
Negative	131 (96%)	5 (4%)	136	
Positive	99 (74%)	34 (26%)	133	***P* = 0.000**
Total	230	39	269	
Presence of extracapsular spread				
Yes	37 (88%)	5 (12%)	42	
No	32 (58%)	23 (42%)	55	***P* = 0.001**
Inconclusive[c]	0 (0%)	2 (6%)	2	
Total	69	30	99	
Presence of non-cohesive invasive front				
Yes	66 (97%)	2 (3%)	68	
No	76 (78%)	22 (22%)	98	***P* = 0.000**
Total	142	24	166	

Presence of lymphovascular invasion				
Yes	31 (89%)	4 (11%)	35	
No	110 (85%)	20 (15%)	130	***P* = 0.7 (NS)**
Inconclusive[c]	2 (100%)	0 (0%)	2	
Total	143	24	167	
Presence of perineural invasion				
Yes	20 (100%)	0 (0%)	20	
No	93 (85%)	17 (15%)	110	***P* = 0.05 (NS)**
Total	113	17	130	
Recurrence				
Yes	49 (93%)	4 (7%)	53	***P* = 0.07 (NS)**
No	184 (84%)	36 (16%)	220	
Total	233	40	273	
Survival status				
Alive	102 (79%)	28 (21%)	130	***P* = 0.002**
Dead	131 (92%)	12 (8%)	143	
Total	233	40	273	

a. Other – includes hypopharynx, pharynx, nasopharynx and unknown primary
b. Unknown – where primary tumour was unknown and disease stage could not be determined
c. Inconclusive – where primary tumour was unknown, surgery was not a modality of treatment or data was missing

.Table 4.5: Characteristics of patients based on HPV status. Significant differences were noted between patients with HPV-positive and negative tumours in social demographics, site of primary tumour, histological grade of tumour differentiation, neck node status, presence of extracapsular spread and non-cohesive invasive front and overall survival.

Head and Neck subsite group	Clinical anatomic location	No. of patients	Type of high-risk HPV
Oral cavity	Dorsum of tongue	1	HPV 16
	Palate	1	HPV 16
	Anterior 1/3 of tongue	1	HPV 16
Oropharynx	Base of tongue	9	HPV 16
	Tonsil	24	HPV 16
Other	Unknown primary	1	HPV 16
	Pharynx	1	HPV 16
	Nares	1	HPV 16
	Nasal cavity	1	HPV 18

Table 4.6: Summary of HPV-positive tumours by anatomic location in the head and neck. The majority of HPV-positive tumours (33/40; 83%) arose from the oropharynx with the tonsils and base of the tongue being the most common subsites of origin. An overwhelming majority of the HPV-positive tumours were infected with high-risk type 16.

4.3.7 Relationship between HPV status and survival

After median follow-up periods of 44 months, a greater proportion of survivors was seen in the HPV-positive group (28/40; 70%) compared to the HPV-negative group (102/233; 44%). Indeed, a Kaplan Meier analysis of overall survival rates demonstrated favourable prognosis for patients with HPV-positive tumours (Figure 4.16). Mean survival time for HPV-positive patients was 63 months (95% CI 54 – 72.8) compared to only 53 months for HPV negative (46.3 – 59.7) (Log Rank (Mantel-Cox) CHI Sq. 7.11 df (1) P = 0.008). The Cox proportional hazards analysis was performed to validate the independent prognostic significance of HPV along with other variables such as age, smoking history, site of primary tumour, disease stage, neck node status and extracapsular spread (all of which were significantly influenced overall survival in univariate analyses). After controlling for age, smoking status, site of primary tumour, disease stage, neck node status and extracapsular spread a positive tumour HPV status was found to be an independent prognostic indicator (HR 0.216; 95% CI 0.06 – 0.771) as was the absence of extracapsular spread (Table 4.7).

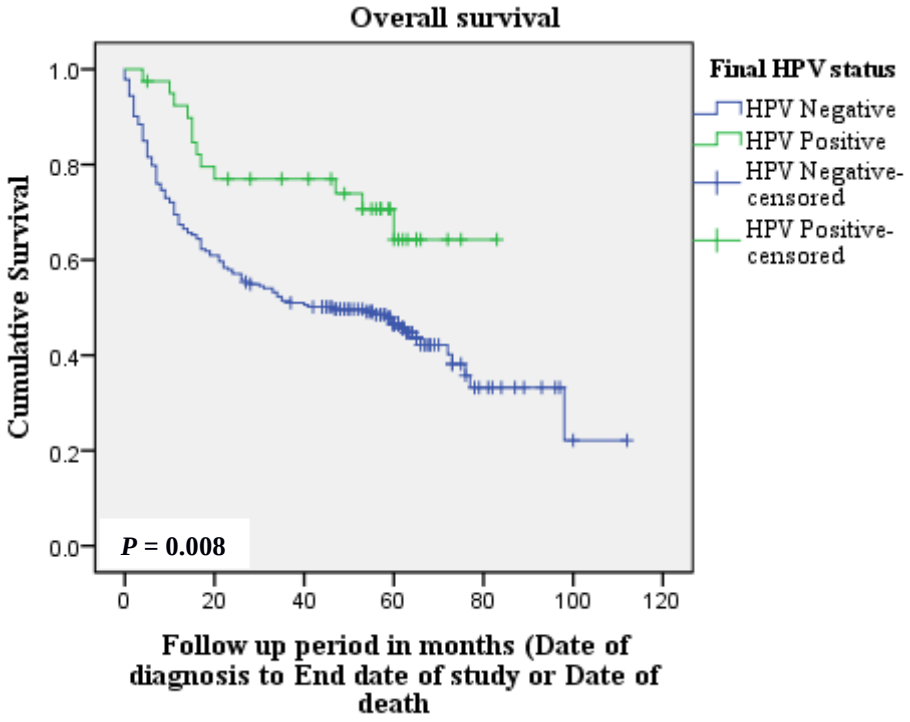

Figure 4.16: Kaplan-Meier plot for overall survival. HPV status of patients in the study was defined by the presence of HPV DNA and p16 overexpression. Four groups of patients were identified; HPV-positive (HPV DNA+ve/p16+ve), HPV-negative (HPV DNA-ve/p16-ve), Equivocal (HPVDNA+ve/p16-ve or HPV DNA-ve/p16+ve). Patients in the cohort with 'Equivocal' status were excluded from the analysis. Overall survival of the HPV-positive group (represented by the green line) was significantly higher than that of the HPV-negative group (represented by the blue line).

Variable	Univariate analysis (P value)	Multivariate analysis – Hazard ratio (95% CI)	P value
Age			
≤ 58	**≤ 0.001**	**(reference category)**	**0.604**
59 – 64		**0.66 (0.207 – 2.103)**	**0.483**
65 – 73		**1.239 (0.542 – 2.832)**	**0.611**
≥ 74		**1.639 (0.555 – 4.839)**	**0.371**
History of smoking			
Non smoker	**0.006**	**(reference category)**	**0.671**
Current smoker		**0.807 (0.300 – 2.170)**	**0.671**
Ex-smoker		**0.595 (0.188 – 1.884)**	**0.378**
Site of primary tumour			
Oral cavity	**0.01**	**(reference category)**	**0.617**
Oropharynx		**1.658 (0.614 – 4.473)**	**0.318**
Larynx		**1.131 (0.456 – 2.805)**	**0.790**
Other		**1.860 (0.630 – 5.497)**	**0.261**
Stage of disease			
Stage I	**≤ 0.001**	**(reference category)**	**0.84**
Stage II		**3.525 (1.088– 11.423)**	**0.36**
Stage III		**1.340 (0.149 – 12.064)**	**0.794**
Stage IVA & IVB			
Extracapsular spread			
Yes	**≤ 0.001**	**(reference category)**	**0.19**
No		**0.321 (0.145 – 0.708)**	**0.005**
Inconclusive		**0.000**	**0.976**
HPV status			
Negative	**0.008**	**(reference category)**	
Positive		**0.216 (0.06 – 0.771)**	**0.018**

Table 4.7: Cox regression analysis for overall survival. On univariate analysis, age, smoking history, site of primary tumour, disease stage, neck node status, extracapsular spread and HPV status were found to be significantly associated with overall survival. After controlling for confounding variables, HPV positivity and extracapsular spread were found to be independent prognostic indicators.

HPV positivity was associated with improved recurrence free survival (Figure 4.17). Mean disease free survival time for HPV-positive patients was 72 months (95% CI 65 – 79) compared to 66 months for HPV-negative patients (95% CI 61 – 72) (Log Rank (Mantel-Cox) CHI Sq. 4.5 (df1) $P = 0.03$).

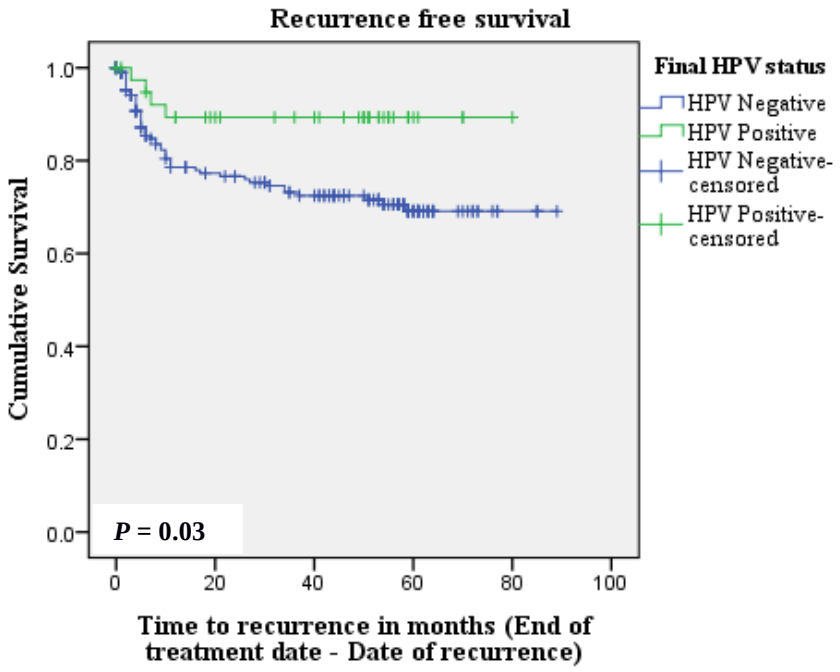

Figure 4.17: Kaplan-Meier plot for recurrence free survival. HPV positivity (n=40) represented by the green line was associated with better disease free survival.

Surprisingly, a positive tumour HPV status did not confer a significant improvement in disease specific survival (Figure 4.18**)**. Mean cause specific survival time for HPV-positive patients was 73 months (95% CI 65.8 – 81.2) compared to 82 months for HPV negative (73.7 – 89.9) (Log Rank (Mantel-Cox) CHI Sq. 2.39 df (1) $P = 0.122$).

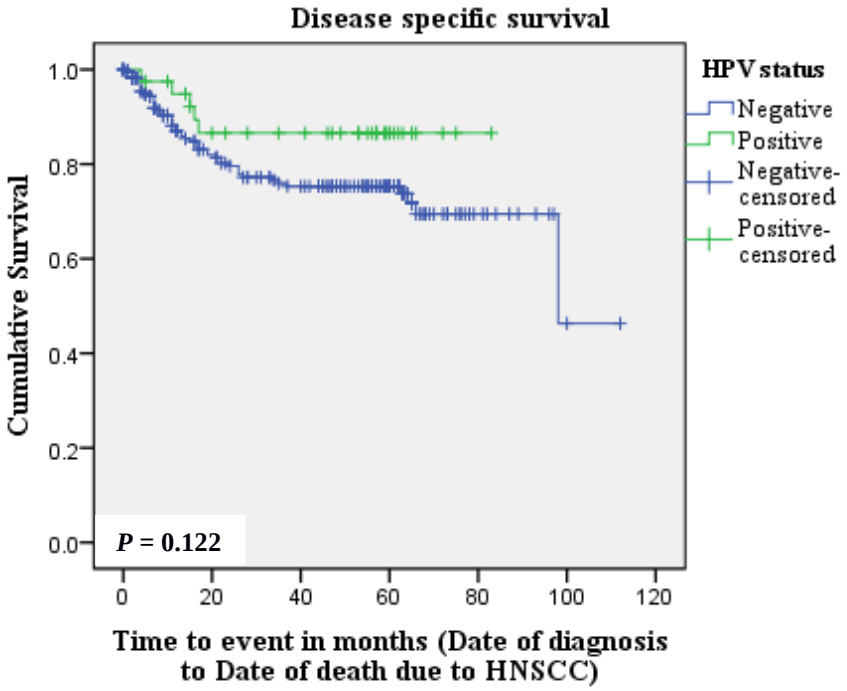

Figure 4.18: Kaplan-Meier plot for disease specific survival. Although patients with HPV-positive tumours (represented by the green line) had better disease specific survival compared to those with HPV-negative tumours (represented by the blue line), it was not found to be statistically significant.

4.3.8 Detection of HPV DNA by *In Situ* Hybridisation

HPV testing in a clinical setting is carried out utilising a variety of tests, most commonly, PCR, p16 immunohistochemistry (IHC) and *In Situ* Hybridisation (ISH). Although consensus is divided over a universally accepted algorithm for HPV testing, a combination of p16 IHC and DNA ISH has been found to be favourable [255]. Given the changes in testing practice trends over the course of this study, a decision was taken to further investigate the HPV status of all 'true positive' study samples by HPV DNA ISH.

Study samples positive for viral DNA by PCR (n = 53) along with 8 negative control samples were tested by DNA ISH at the Department of Clinical Pathology in Ninewells Hospital. The test results were interpreted by two independent observers (AS and SW). Observations were compared and differences in opinions were reconciled. Surprisingly, only 29 out of 53 (55%) samples tested positive for HPV by DNA ISH. Furthermore, only 24 samples were positive for viral DNA by PCR and p16. Out of 24 negative samples, 8 were positive for HPV DNA by PCR and 16 samples were positive for both p16 and HPV DNA by PCR. All tissue samples sent as negative controls were also negative for HPV by ISH. Figures 4.19 (A), (B), (C) and (D) represent ISH detection of

HPV in specimens 88, 45, 137 and 161 respectively.

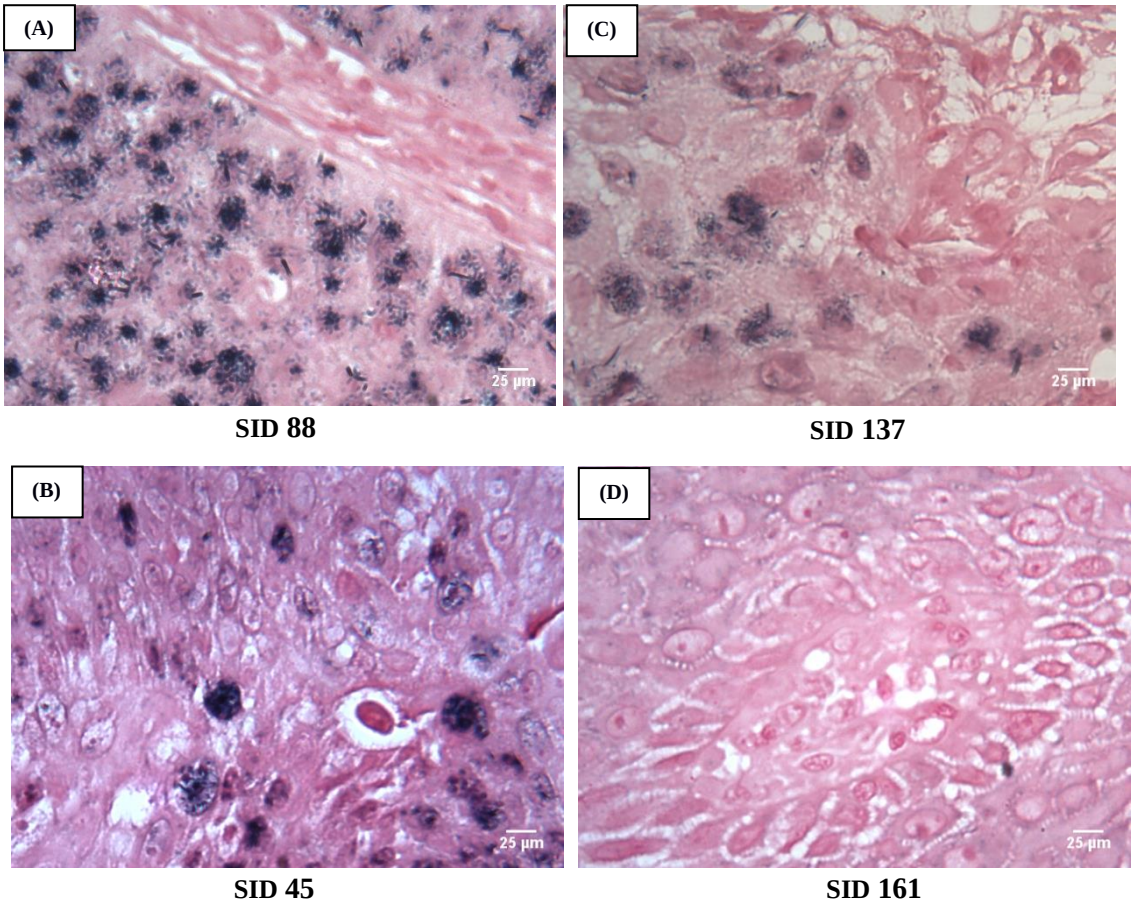

Figure 4.19: Detection of high-risk HPV by DNA ISH in histological sections of cohort specimens. (A) Poorly differentiated squamous cell carcinoma involving the dorsum of the tongue (x400) demonstrating HPV staining in tumour cell nuclei. (B) ISH positive for HPV in a poorly differentiated squamous cell carcinoma involving the anterior 2/3 of the tongue (x400) (C) Oropharyngeal squamous cell carcinoma demonstrating dot like ISH signal for HPV (D) Squamous cell carcinoma of the tonsil negative for HPV ISH.

4.4 Discussion

The research presented in this chapter was undertaken to establish the prevalence of HPV in a cohort of HNSCC patients in Tayside, Scotland. This study involved a retrospective analysis of archived FFPE tumour biopsy specimens obtained from the local tissue bank. Although FFPE tissue is an invaluable resource for obtaining nucleic acids - especially DNA for molecular analyses - its isolation is often challenging. Formalin fixation leads to the generation of nucleic acid protein cross linkages and can greatly affect DNA quality. Furthermore, temperature and pH of a fixative, when not adequately controlled, can adversely affect the quality of DNA leading to its degradation [97].

Preliminary experiments were carried out to determine the best approach for optimal recovery of DNA from the archived tissue. Two protocols; the QIAamp FFPE DNA extraction kit and an in-house DNA lysis buffer (LC) were compared. DNA concentration and total yield obtained with buffer LC was consistently higher than that obtained with the Qiagen kit. While the LC buffer protocol employs a simple ethanol precipitation purification step, the Qiagen kit uses silica based spin columns for nucleic acid purification. Multi-step processing of lysates and loss of DNA in the silica spin columns may explain the apparent low DNA yield with the Qiagen kit [182]. Absorbance ratio A260/230 values were often very low or variable with both protocols indicating either a problem with the tissues or contamination with organic compounds or solvents (such as phenol and glycogen) utilised in the extraction process [286]. However, comparison of PCR results confirmed that template DNA isolated with the Qiagen kit was more amplifiable than that obtained with the in-house protocol as observed in previously published data [273].

Ever since a causal association between high-risk HPV and HNSCC was first proposed by Syranjen *et al*., [294] the presence of HPV DNA in HNSCC has been reported by numerous groups [218], [13], [99]. Previous studies exploring prevalence of HPV in the British population have focused on oropharyngeal cancers alone and where heterogeneous cohorts were selected, the sample sizes were small [264], [75], [321], [4]. In effect, this study is perhaps the first to investigate the prevalence of HPV in a large heterogeneous Scottish cohort of squamous cell carcinomas of the head and neck. High-risk HPV DNA was detected in 18% of the study sample. Previously, Maitland *et al*. [185] and Anderson *et al*. [4] have investigated HPV DNA in small heterogeneous cohorts in the English and Scottish populations respectively. While HPV DNA was detected in 46% of oral squamous biopsies and normal oral tissues by Maitland *et al*., Anderson *et al*. reported HPV DNA in 10% of the population. A systematic review by Kreimer *et al*. looking at the prevalence of HPV worldwide reported an overall prevalence of 25% [148]. While the findings of this study are somewhat similar to those of other studies, there exists some variability in reported results of prevalence. This variability may be attributed to differences in sample sizes, variability in sample sources (fresh frozen vs FFPE) and the use of different primer sets and probes for detection of the virus [148]. The vast majority of viral genotypes detected in this study were HPV type 16, with other high-risk HPV types 18 and 33 comprising much smaller proportions. These findings are consistent with results reported by the other studies

[148], [101]. Oropharyngeal tumours comprised 20% of the study sample and more than half of the OPSCCs tested positive for high-risk HPV type 16. These statistics are in line with other studies conducted on the British population [321], [131], [4], but higher than the European average of 28% [148].

Immunohistochemical validation of p16 overexpression as a surrogate marker for HPV-related HNSCCs, especially OPSCC, has been widely documented [282], [318]. Expression of the E7 viral oncoprotein leads to functional inactivation of pRB, a negative regulator of the p16INK4A tumour suppressor gene product, resulting in subsequent overexpression of the p16 protein. Consequently, high-risk HPV-associated head and neck tumours often demonstrate nuclear and cytoplasmic p16 overexpression, a histological feature predominantly absent in HPV-negative tumours. Tumour samples for this study were selected based on the availability of a p16 status. Accordingly, 16% of the study cohort was p16-positive while the remaining 84% was p16-negative. Similar results were reported by Larsen *et al*. in a pooled analysis of 3625 patients where p16 overexpression (reported as staining equal to or exceeding 70% of the cells) was seen in 19.5% (n = 764) of the subjects [157]. However, it must be noted here that the findings of this study are based on a cohort of heterogeneous tumours while that of Larsen *et al*. are based predominantly on tumours arising in the oropharynx. In this study, a comparison of baseline clinical characteristics between patients with p16-positive and negative tumours indicated that those with p16-positive tumours were younger and were more likely to be non-smokers at diagnosis. Patients with p16-negative tumours were more likely to present with a history of heavy drinking at diagnosis. Assessments of histopathological features show that p16-positive tumours were mostly poorly or moderate to poorly differentiated tumours arising predominantly in the oropharynx. Although patients with p16-positive tumours presented with late stage disease, they had better overall and recurrence free survival rates. HPV/p16 positive tumours have been recognised by previous studies as a clinically and morphologically distinct subgroup [322]. While the results presented in this section are based on tumour p16 status alone, the findings are consistent with those reported by other studies [289], [318].

Although data suggests that p16 overexpression is an independent prognostic predictor in the tonsillar subset of HNSCC [154], its efficiency as a single test for detection of high-risk HPV in HNSCCs is debatable [283]. However, p16 immunostaining, when

used in conjunction with viral DNA detection methods has been shown to have optimal sensitivity and specificity [283], [93]. In this study, samples were initially screened and selected based on p16 status and followed up with HPV DNA detection by PCR using a combination of consensus and type specific primers. Four categories of tumours were identified, HPV DNA-ve/p16-ve or 'True negative', HPV DNA-ve/p16+ve or 'Equivocal', HPV DNA +ve/p16-ve or 'Equivocal' and HPV DNA +ve/p16+ve or 'True positive'. Incongruent p16 and HPV DNA testing results noted in 7% of the study population is similar to that reported by Evans *et al.* suggesting that neither approach is a reliable standalone for accurate reporting of HPV infection in HNSCC [75]. Overexpression of p16 in the absence of viral DNA was noted primarily in non-oropharyngeal sites and suggests that p16 IHC may not be a reliable marker of HPV infection in tumours arising in sites other than the oropharynx [32]. It may also be indicative of an alternate mechanism of disruption of the pRB pathway [189]. On the other hand samples that were HPV DNA +ve/p16-ve may be indicative of a biologically irrelevant HPV infection [256].

High-risk HPV infection was identified in 14% of the study population and is higher than the prevalence reported in a previous study conducted on a heterogeneous cohort in the South East of Scotland [4]. Consistent with reports of previous studies, HPV-positive patients in this study cohort were younger with no history of smoking at diagnosis [291], [321]. HPV infection is the most commonly transmitted sexual infection and occurs through direct skin or mucosal contact [309]. The apparent social demographics of patients with HPV-related HNSCC may be attributed to changes in lifestyle practices, for example, higher number of oral sex partners or first sexual encounter at a younger age along with a fall in tobacco use over the recent years [189], [54]. Amongst the head and neck anatomic subsites, the oropharynx is especially susceptible to infection with high-risk HPV [99], a finding consistent with this study. High-risk HPV was detected in only three oral cavity tumours and none of the laryngeal tumours were positive for the virus. The oropharynx, particularly the lingual and palatine tonsils, is lined by reticulated epithelium which may be more prone to infection with HPV. By inhibiting virus-specific T-cells, and thereby allowing the virus to evade an immune response during initial infection and subsequent malignant transformation, the reticulated epithelium of the oropharynx probably offers an immune privileged site for initial infection and subsequent malignant transformation. In addition, the natural breaks in the reticulated epithelium leave the basement membrane exposed to deposition

of the virus [324]. Generally, patients with HPV-associated OPSCC present with Stage III or IV disease and cystic nodal metastasis has been reported as a prominent feature of tonsillar squamous cell carcinomas [189], [103]. Indeed, patients with HPV-related tumours in this cohort were diagnosed with Stage IV disease and nodal metastasis. A closer look at the HPV-positive oropharyngeal tumours in this study revealed that the vast majority arose in tonsils followed by base of tongue. In addition to social demographics and clinical characteristics of patients with HPV-related HNSCC, this study also reports findings on histological features of the tumour samples. Interestingly, the majority of HPV-positive tumours were poorly differentiated and were more likely not to exhibit extracapsular spread and presence of a non-cohesive invasive front. Although HPV-positive tumours have been widely classified as poorly differentiated owing to the divergence in appearance of tumour cells from the stratified epithelium that lines the oropharynx, such tumours arise in the tonsillar crypts and tumour cells tend to resemble cells of the reticulated epithelium from where they arise. In a recent review article, the author recommends classification of HPV-positive tumours as "well differentiated" as it is perhaps more appropriate and is in keeping with tissue morphology and expected clinical behaviour of this subset of HNSCC [323]. Absence of extracapsular spread and a non-cohesive invasive front may also be attributed to the non-aggressive nature of these tumours.

The impact of HPV as predictive marker for improved treatment outcomes and survival has been established by numerous studies [79], [158], [253]. In this study, HPV-positive patients had improved overall survival (70%) compared to HPV-negative patients (44%). This was similar to the overall survival rates noted for p16-positive patients (68%). Likewise, mean recurrence free survival time for HPV-positive patients was significantly improved compared to that of HPV-negative patients. The results related to survival rates from this study are similar to other studies, albeit slightly lower [321], [4] which may be attributed to differences in study cohorts (heterogeneous vs oropharyngeal). Interestingly, HPV positivity did not confer a significant improvement in disease specific survival even though it was discernible in the Kaplan Meier plot (Figure 4.18). Similar findings were noted for patients with p16 positive tumours. The findings of studies reporting on the relationship between HPV status and cause specific survival are varied. Wells *et al.* found a significant association between HPV status and cause specific survival in a cohort of 60 patients and similar findings were reported by Dahlgren *et al.* [321], [57]. However, both studies were carried out on a cohort of

oropharyngeal/tonsillar squamous cell carcinomas and fresh frozen biopsy specimens were employed in the study by Dahlgren *et al.*. In a large mixed cohort study, employing fresh frozen biopsy specimens, conducted by Gillison *et al.*, HPV-positive HNSCC patients were estimated to have significantly reduced risk of death from cancer compared with those with HPV-negative tumours after adjusting for confounding factors [101]. The differences in cause specific survival between this study and others may be attributed to differences in cohort type and size and in percentages of deaths due to cancer. Additionally, disease specific survival curves can often be misleading as they do not include death caused by disease related factors such as treatment [252]. Ritchie *et al.*, examined the relationship between survival and HPV status in a mixed cohort of 141 patients and found that patients with HPV-positive tumours also tended to have better disease-specific survival than those with HPV-negative tumours among all carcinomas but this association was not statistically significant, a finding concurrent with this study [254].

As the practice trends in diagnostic HPV testing have evolved over the course of this study, a decision was taken to test all the samples in this study positive for HPV DNA with DNA ISH. Interestingly, only a little more than half of the samples (55%) tested positive for HPV by ISH and fewer still for HPV DNA by PCR, p16 and DNA ISH culminating in an overall HPV prevalence of 8%. ISH differs from PCR and p16 IHC in that it allows for detection of HPV DNA within the nuclei of infected tumour cells. Additionally, the physical state of the virus as indicated by punctate staining for integrated virus and diffuse staining for episomal virus is also evident with ISH [310]. However, a major drawback of the ISH is its lack of sensitivity as it is only able to detect HPV when there are around 10 copies of virus per cell [310]. Indeed, Schache *et al.* investigated the sensitivity, specificity and prognostic significance of ISH among other techniques by comparing it to the 'gold standard' RNA qPCR and found that ISH demonstrated only 88% sensitivity and conferred the least prognostic significance [264].

4.5 Conclusion

In this study, tumour samples were defined as HPV-positive only if they were positive for both HPV DNA and p16. As such, the study cohort was grouped into 3 classes; true HPV-positive (HPV DNA+ve/p16+ve), HPV-negative (HPV DNA-ve/p16-ve) and Equivocal (HPV DNA+ve/p16-ve or HPV DNA-ve/p16+ve). High-risk HPV was

prevalent in 14% of the sample population with HPV type 16 being the most predominant genotype. An overwhelming majority of HPV-positive tumours were oropharyngeal SCCs arising in the ‘Tonsils’ and ‘Base of Tongue’. The clinical characteristics of patients with HPV-positive and negative tumours and associated histopathological features were consistent with that reported in the literature. Thus, the findings reported in this chapter of the thesis contribute to existing knowledge that patients with HPV positive tumours are a clinically and demographically distinct patient population with a clear survival advantage afforded by a positive tumour HPV status.

Chapter 5

INVESTIGATION OF EBP50 AS A POTENTIAL NOVEL MARKER OF HPV-ASSOCIATED HNSCC

Ezrin-Radixin-Moesin Binding Phosphoprotein 50 (EBP50), a PDZ domain scaffolding protein, is found in abundance at the plasma membranes of polarised epithelial cells where these proteins form complexes that regulate tissue architecture and cell migration. While our lab has previously shown that EBP50 is a novel target of the high-risk HPV E6 oncoprotein [237], there is very little published work in this area with the 2011 work of Accardi *et al*., on the coordinated degradation of EBP50 by E6 and E7 oncoproteins of high-risk-HPV type 16, being possibly the only published report to date of direct relevance [2]. Moreover, even though expression patterns of EBP50 and its clinical significance have been studied previously in colorectal cancer [186], [188] and breast cancer [187], studies for HNSCC have not been conducted. This, in addition to the knowledge that high-risk HPV is an important risk factor for certain types of HNSCC [326], [218] prompted an investigation into the distribution of EBP50 protein in HPV-associated HNSCC tumour specimens from a heterogeneous study cohort. This chapter which will focus initially on EBP50 staining patterns obtained by immunohistochemical analysis of randomly selected specimens, followed by a detailed assessment of distribution patterns of the protein in HPV- positive and HPV- negative specimens.

5.1 Selection of anti-EBP50 antibody and its optimisation

Immunostaining of tissue sections from the study cohort was carried out utilising a polyclonal anti-EBP50 antibody according to the protocol outlined in Chapter 2 Section 2.9. Preliminary experiments to determine optimal antibody dilutions were carried out on normal oral mucosa FFPE sections using antibody dilutions recommended by the manufacturer (10 µg/ml) as a guideline. The resulting staining was very strong with a high background. Additional antibody titrations at higher dilutions (1:1000, 1:1500. 1:2000 and 1:3000) were set up along with varying incubation times (overnight, 60 min and 40 min) and H_2O_2 concentrations. Optimal EBP50 staining with minimal background was obtained with an antibody dilution of 1:2000 after 40 min incubation at room temperature. Figure 5.1 (A), (B) demonstrate strong EBP50 membrane and cytoplasmic staining at an antibody dilution of 1:3000 with overnight and 60 min incubation times respectively and images (C), (D) show EBP50 membrane staining at

antibody dilutions of 1:2000 with 60 and 40 min incubations respectively .

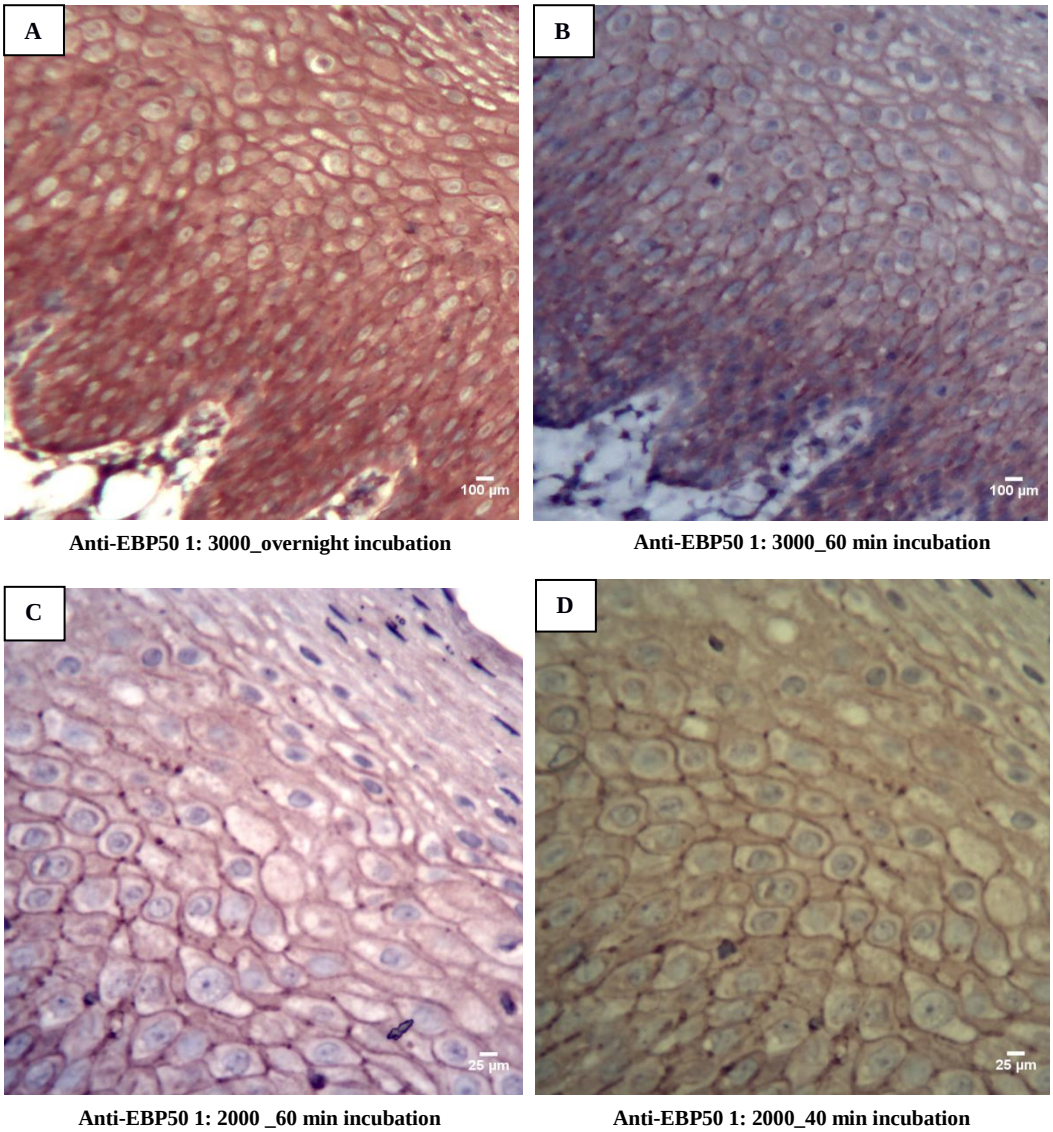

Figure 5.1: Optimisation of antibody dilution. FFPE sections of normal oral mucosa were incubated with anti- EBP50 antibody (PA1-090, Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leics, LE11 5RG, UK) at dilutions of 1:1000, 1:1500, 1:2000 and 1:3000 (A) / (B) EBP50 immunoreactivity in sections incubated with the primary antibody at 1:3000 dilution overnight and 60 min respectively. A higher level of non-specific background staining was observed with overnight incubation and EBP50 signal was found to be slightly weak with one hour incubation (Magnification x100); (C) / (D) EBP50 immunoreactivity in sections incubated for one hour and 40 min respectively with the primary antibody dilutions of 1:2000. Although a higher dilution of 1:2000 did not completely eliminate non-specific background staining, EBP50 staining in the cytoplasm and plasma membranes were found to be optimal at this dilution (Magnification x400).

In order to confirm specificity of the polyclonal antibody PA1-090 for the target protein EBP50, an antibody blocking assay was performed. As the immunising peptide for the commercial antibody employed in this study was unavailable for purchase, the commercial antibody was blocked with an 'in-house' Glutathione S Transferase (GST) tagged recombinant EBP50 protein. Tissue sections were incubated with primary anti-

EBP50 antibody alone (Figure 5.2 (B)), anti-EBP50 antibody with added GST protein (2 µg) and secondary antibody alone a controls (Figure 5.2 (C), (A) and anti-EBP50 antibody with added GST-EBP50 protein (2 µg or 5 µg) incubated either for 20 min or overnight (Figure 5.2 (D), (E), & (F)). EBP50 membrane and cytoplasmic signals were detected with both primary antibody only and primary antibody with added GST. Shorter incubation with primary antibody and GST-EBP50 mixture merely resulted in a reduction in the intensity of the signal. However, a longer overnight incubation with the antibody-recombinant protein mixture resulted in much of the antibody being absorbed and a complete signal block was achieved, thereby confirming that the primary antibody PA1-090 was indeed specific for EBP50.

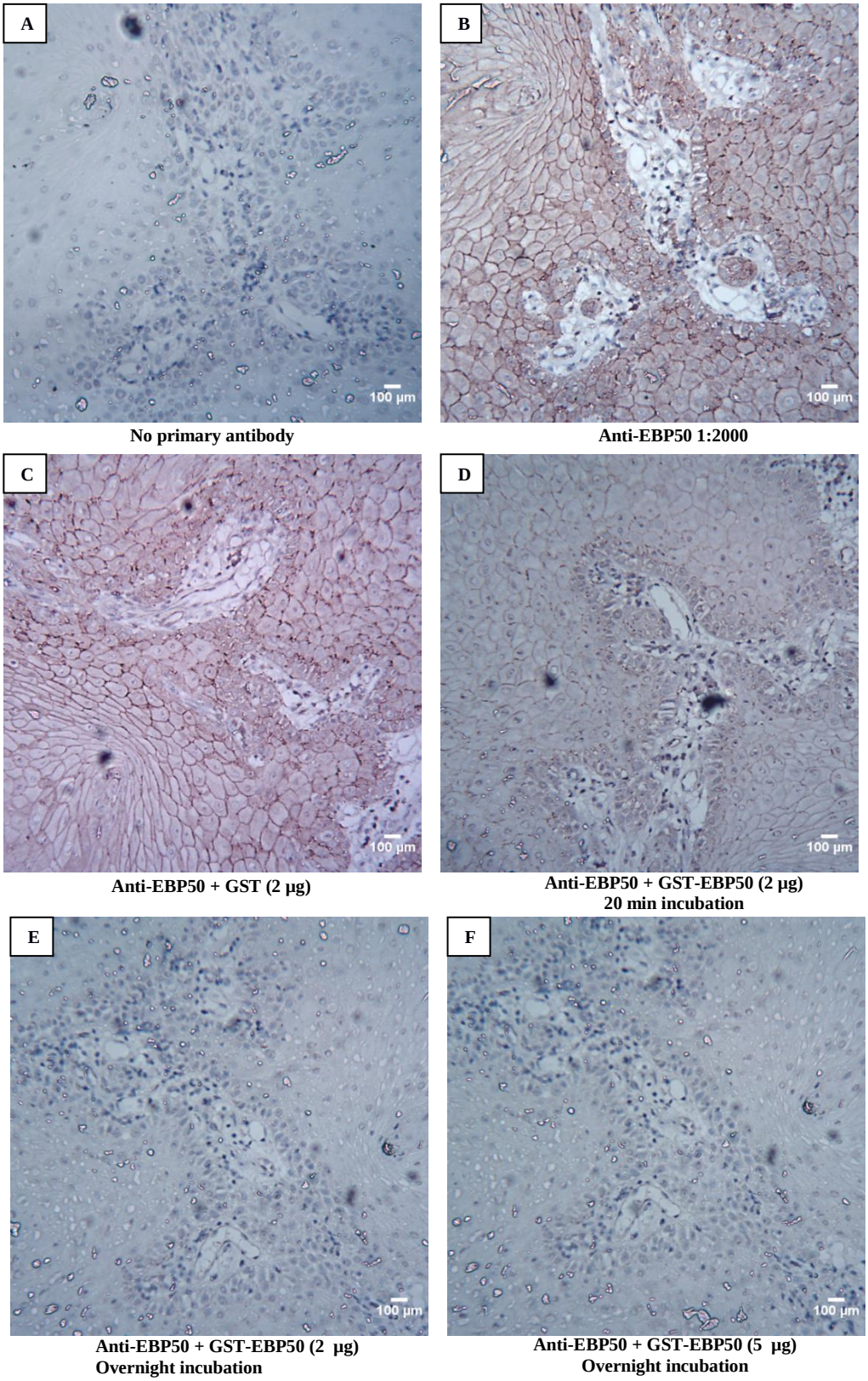

Figure 5.2: Antibody neutralisation assay to determine specificity of the primary antibody. The commercial primary antibody PA1-090 was neutralised with GST tagged EBP50 (A) FFPE section incubated with secondary antibody showing negative EBP50 expression; (B) / (C) sections incubated with primary antibody PA1-090 alone and GST + PA1-090 respectively. Membrane and cytoplasmic EBP50 expression was detected in both; (D), (E) & (F) EBP50 expression in sections following incubation with neutralised primary antibody (+ GST-EBP50 2 µg) for 20 min, overnight and primary antibody (+ GST-EBP50 5 µg) overnight respectively. Complete blocking of primary antibody was achieved following overnight incubation with blocking peptide (Magnification x 100).

5.2 EBP50 expression in normal oral mucosa

Epithelial cells, along with tight junctions, form a highly specialised polarised barrier separating the apical cell surface from the basolateral membranes and disruption of this polarity appears to be an early event in the development of human epithelial tumours [209]. EBP50 localises at the apical membranes of polarised human epithelial cells where it promotes the maintenance of normal tissue architecture by forming complexes with other cell polarity proteins [206]. The loss of EBP50 expression at apical membranes, its distribution to the cytoplasm and nuclear overexpression have all been observed in several types of human cancer [95]. Since procuring normal oral mucosa for this study was challenging, a 'normal' tonsil specimen was obtained for immunohistochemical analysis of EBP50 distribution. EBP50 expression patterns observed in the tonsil tissue served as a baseline for comparison of protein distribution. Membrane or cytoplasmic EBP50 expression was detected in different layers of the normal oral epithelium. Figure 5.3 is a representative image of EBP50 staining in a tonsil specimen. EBP50 expression was predominantly membranous in suprabasal layers of the epithelium (Figure 5.3 (B) & (D)). No immunoreactivity was detected in the Stratum Corneum. A gradual shift in EBP50 expression from membranous to predominantly cytoplasmic was observed from suprabasal to the basal layers of the epithelium (Figure (B) & (C)). No nuclear EBP50 immunoreactivity was detected.

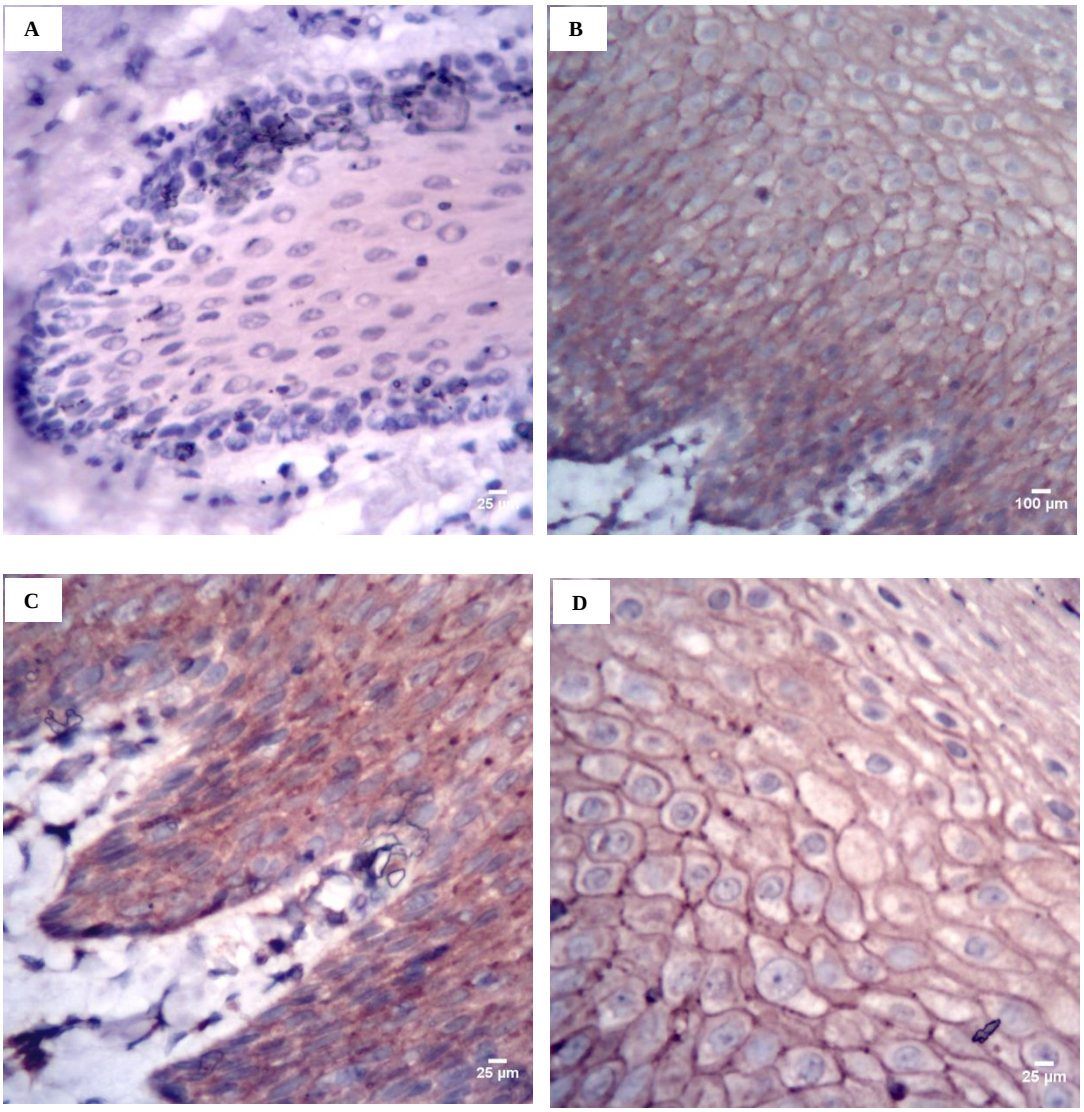

Figure 5.3 EBP50 expression in normal oral epithelium (A) section stained with secondary antibody only showing negative EBP50 expression (Magnification x400); (B) section stained with anti-EBP50 antibody (Magnification x100); (C) basal cells of the epithelium demonstrating cytoplasmic EBP50 expression with no membrane immunoreactivity (Magnification x400); (D) EBP50 expression was seen predominantly in the plasma membranes of cells in the suprabasal layers of normal oral epithelium (Magnification x 400).

5.3 EBP50 expression in primary head and neck squamous cell carcinomas

In order to study EBP50 expression in primary squamous cell tumours of the upper aerodigestive tract, 156 specimens were selected from the main study cohort. This smaller cohort included all samples positive for HPV DNA along with a random selection of HPV DNA negative specimens from different anatomic sites of origin. Sections of 5 µm thickness were mounted on slides and stained for EBP50 according to the protocol detailed in Section 2.9 of Chapter 2. The resulting cytoplasmic and membrane EBP50 expression patterns were analysed under a light microscope and

grouped into 4 categories, each of which is described in detail in the subsections below.

5.3.1 Expression pattern 1: Predominantly cytoplasmic with absence of EBP50 expression in the plasma membranes of epithelial cells

The most common expression pattern observed in the cohort was predominantly cytoplasmic. Unlike normal oral mucosal epithelial cells, EBP50 expression in the plasma membranes of tumour cells was completely absent. Cytoplasmic staining was of a similar intensity to that observed in the basal layers of normal oral mucosal epithelium. Figure 5.4 is a representation of membrane excluded and 'predominantly cytoplasmic' type of EBP50 expression in a poorly differentiated tumour arising in the oral cavity. Membranous EBP50 expression is evidently absent in two different tumour cell fields of the specimen (Figure 5.4 (D) & (F)).

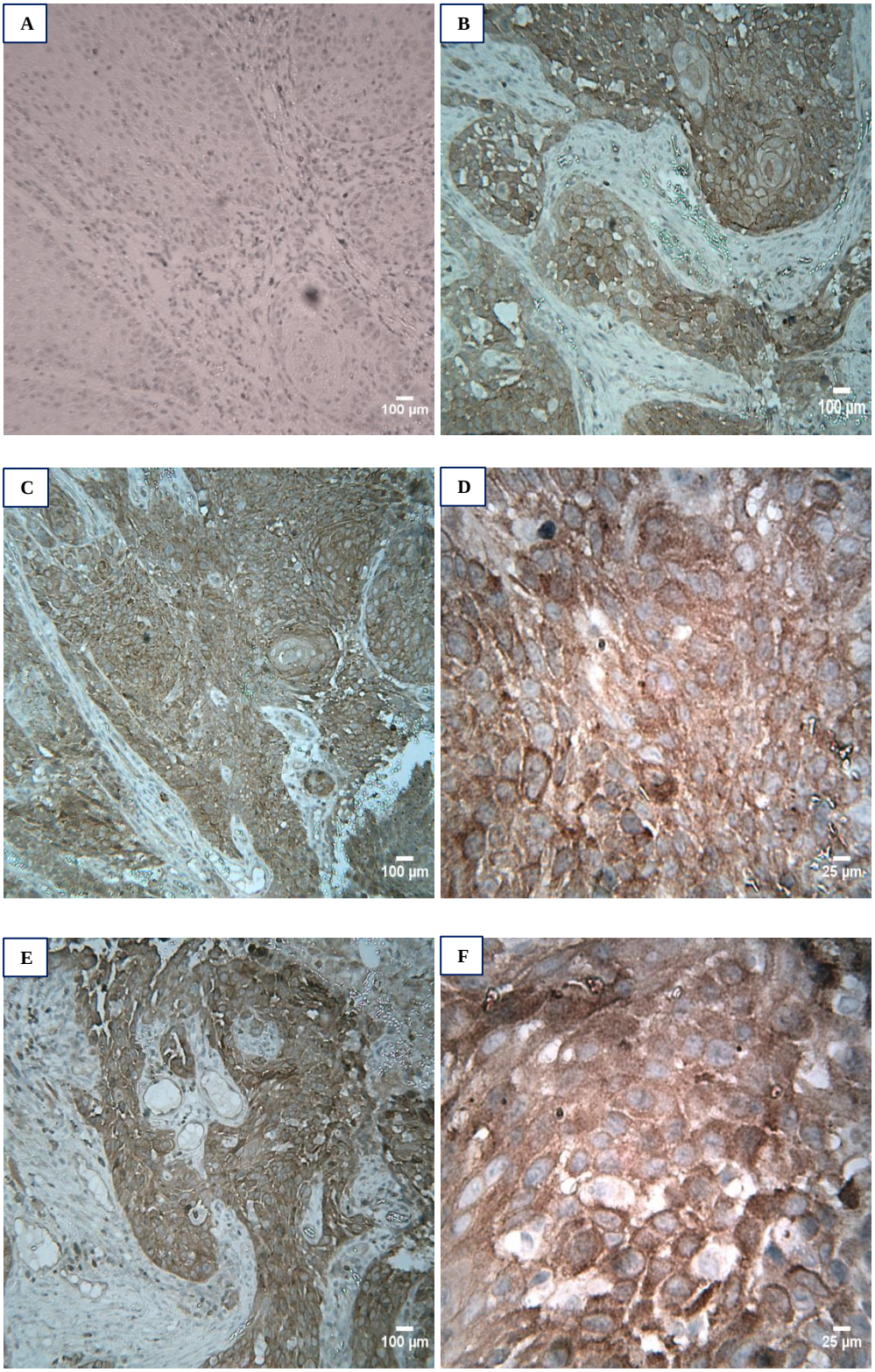

Figure 5.4: 'Predominantly cytoplasmic' type of EBP50 expression (SID 87) (A) section of OSCC stained with secondary antibody only showing negative EBP50 expression (Magnification x100); (B) FFPE section of a poorly differentiated tumour involving the retromolar trigone stained with anti-EBP50 antibody (Magnification x100); (C) / (E) Random fields of the section showing cytoplasmic EBP50 expression in the tumour cells. No immunoreactivity for EBP50 was detected in the plasma membranes of the cells (Magnification x100); (D) / (F) Images of (C) / (E) respectively at higher magnification (x400).

5.3.2 Expression pattern 2: Weak or negligible cytoplasmic EBP50 expression

Weak cytoplasmic staining was the second most commonly recurring distribution pattern observed in the cohort. Decreased EBP50 expression was evident in the cytoplasm of tumour epithelial cells, thereby resulting in weak staining or almost negligible immunoreactivity in some specimens. Decreased cytoplasmic signal was also accompanied by an absence of membranous EBP50 expression similar to the first staining pattern. Figure 5.5 is a representation of 'Weak/negligible cytoplasmic' type of EBP50 expression. Decreased cytoplasmic staining and lack of membrane expression can be visualised in Figure 5.5 (D) and (F).

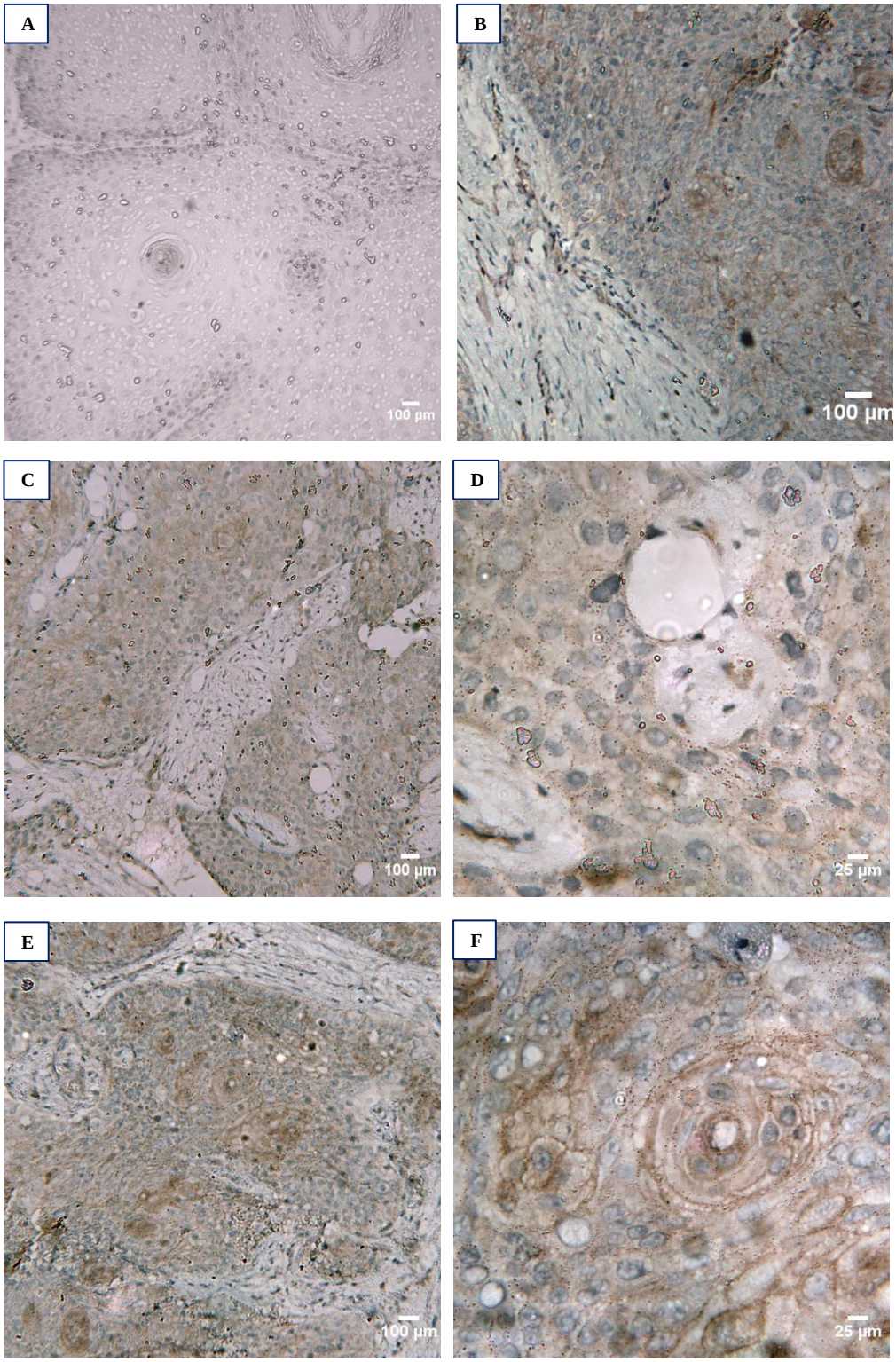

Figure 5.5: 'Weak/negligible' type of EBP50 expression (SID 137) (A) section of OSCC stained with secondary antibody only showing negative EBP50 expression (Magnification x100); (B) FFPE section of a tumour involving the base of tongue stained with anti-EBP50 antibody (Magnification x100); (C) / (E) Random fields of the section showing weak or negligible cytoplasmic EBP50 expression in a majority of the tumour cells. No immunoreactivity for EBP50 was detected in the plasma membranes of the cells (Magnification x100); (D) / F) Images of (C) / (E) respectively at higher magnification (x400).

5.3.3 Expression patterns 3 & 4: Mixed EBP50 distribution and other less common expression patterns

A third less frequently recurring heterogeneous EBP50 expression pattern was observed in a few specimens wherein EBP50 could be detected in the plasma membranes of a few tumour cells while it was either absent or weak in the others. Likewise, a few specimens demonstrated mixed cytoplasmic expression of EBP50 ranging from clear positive to weak or negligible immunoreactivity. Figure 5.6 is a representation of mixed or 'Heterogeneous' EBP50 expression in a moderately differentiated squamous cell carcinoma of the buccal mucosa. While EBP50 expression was predominantly cytoplasmic, membranous immunoreactivity ranging from weak/negligible to strong was detected in a few groups of tumour cells (Figure 5.6 (D), (F).

Other less common staining patterns observed in a few samples in the cohort included strong cytoplasmic expression of EBP50 with no immunoreactivity in the plasma membranes of tumour cells (Figure 5.7).

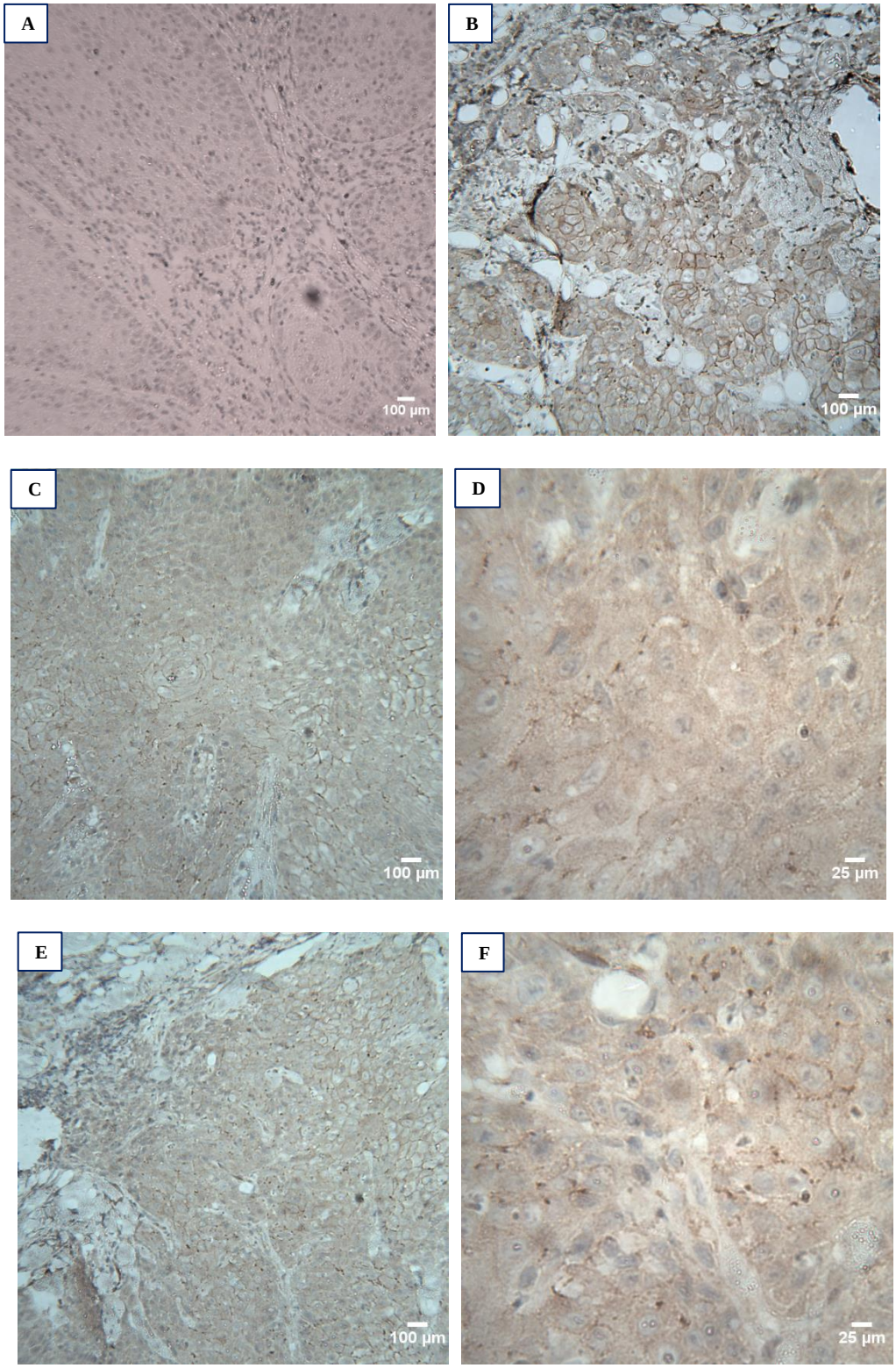

Figure 5.6: 'Heterogeneous' type of EBP50 expression (SID 89) (A) section of OSCC stained with secondary antibody only showing negative EBP50 expression (Magnification x100); (B) FFPE section of a moderately differentiated tumour of the buccal mucosa stained with anti-EBP50 antibody (Magnification x100); (C) / (E) Random fields of the section showing positive staining for EBP50 in the cytoplasm of tumour cells and mixed EBP50 expression ranging from weak to positive in the plasma membranes (Magnification x100); (D) / (F) Images of C/E respectively at higher magnification (x 400).

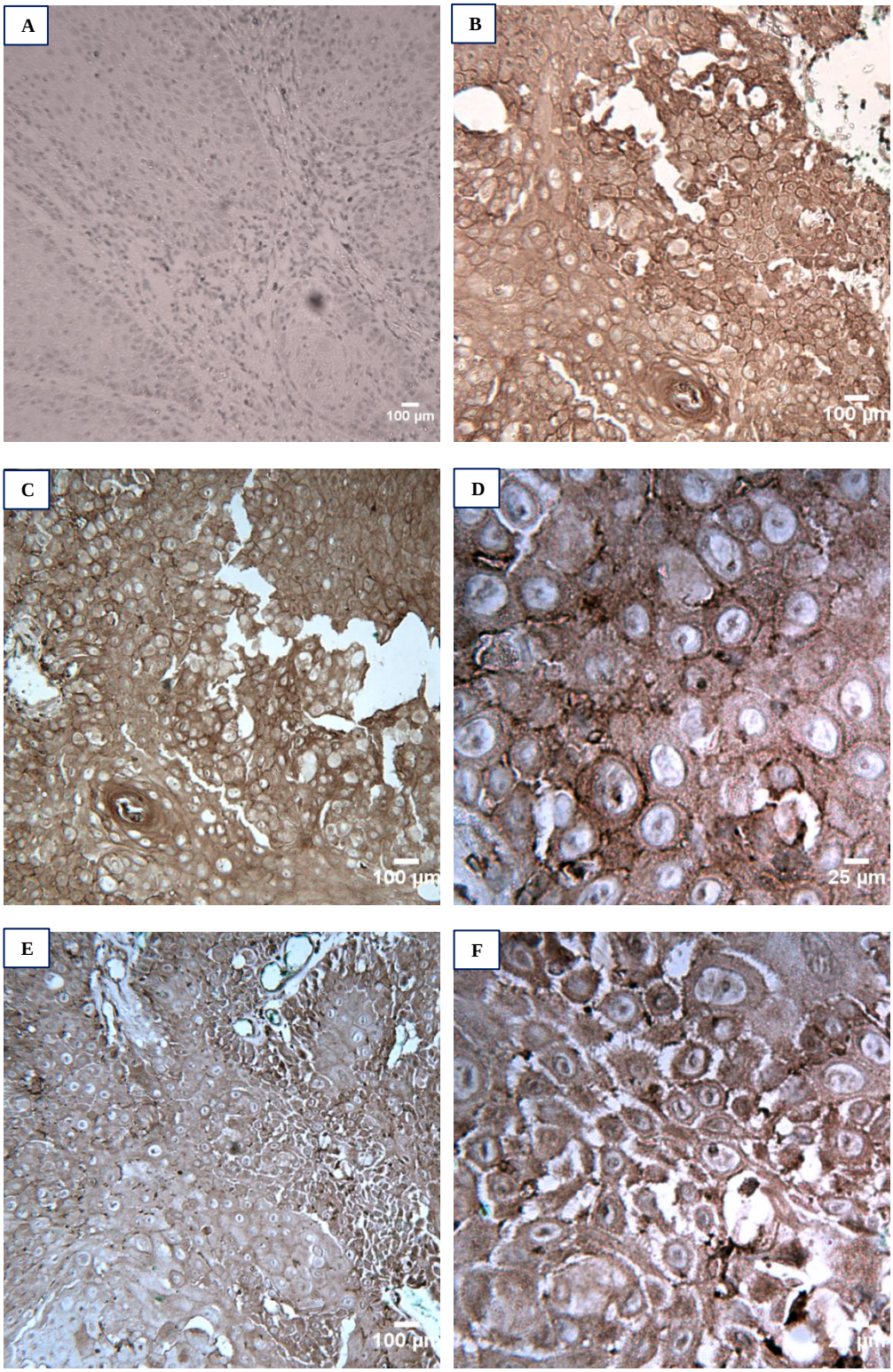

Figure 5.7: 'Other' type of EBP50 expression (SID 274) (A) section of OSCC stained with secondary antibody only showing negative EBP50 expression (Magnification x100); (B) FFPE section of a moderately differentiated laryngeal tumour stained with anti-EBP50 antibody (Magnification x100); (C) / (E) Random fields of the section showing strong EBP50 expression in the cytoplasm of tumour cells. No membrane EBP50 immunoreactivity was detected (Magnification x100); (D) / (F) Images of D/G respectively at higher magnification (x 400).

5.4 Implications of EBP50 expression in HNSCC

The EBP50 expression patterns identified through IHC analysis of tumour specimens in the study cohort were classified as 'Predominantly cytoplasmic (Membrane excluded)', 'Weak/Negligible cytoplasmic (Membrane excluded)', 'Heterogeneous (mixed membranous and/or cytoplasmic)' and 'Other'. Out of 156 specimens, 'predominantly cytoplasmic' EBP50 expression was detected in 76 (49%) samples, 'Weak/negligible cytoplasmic' pattern in 44 (28%), 'heterogeneous' and 'other' patterns in 26 (17%) and 10 (6%) samples respectively.

5.4.1 EBP50 expression and clinicopathological characteristics of the study cohort

The clinical and pathological characteristics of the study subset were subsequently analysed based on EBP50 expression patterns (Table 5.1). It was noted that smoking status and disease stage were significantly associated EBP50 expression. While tumour samples of 'smokers' demonstrated 'predominantly cytoplasmic' EBP50 expression, 'non-smokers' and ex-smokers were more likely to have tumours exhibiting with 'weak/negligible cytoplasmic' EBP50 expression ($P = 0.019$). Nearly three-quarters of the patients (73%) with tumours expressing 'weak/negligible cytoplasmic' EBP50 also presented with late stage disease ($P = 0.02$). Analysis of the tumour histopathology of patients in the study subset showed that the majority of tumours with 'weak/negligible cytoplasmic' EBP50 expression (71%) originated in the oropharynx while those with 'predominantly cytoplasmic' or heterogeneous EBP50 distribution arose in the oral cavity and the larynx ($P < 0.001$). Interestingly, patients with tumours that demonstrated 'weak/negligible cytoplasmic' EBP50 expression were also more likely to have positive neck nodes, overexpression of p16 and positive tumour HPV status ($P < 0.001$). EBP50 expression patterns were not found to have significant associations with grade of tumour differentiation, alcohol consumption, overall survival or recurrence.

Variable	EBP50 staining patterns					
	Predominantly cytoplasmic expression	**Weak/negligible cytoplasmic expression**	**Hetero-geneous expression**	**Other**	**Total**	**Pearson's CHI Square**
History of smoking						
Non-smoker	9 (15%)	12 (32%)	5 (22%)	1 (11%)	27	
Smoker	36 (60%)	8 (22%)	11 (48%)	6 (67%)	61	***P* = 0.019**
Ex-smoker	15 (25%)	17 (46%)	7 (30%)	2 (22%)	41	
Total	60	37	23	9	129[a]	
History of alcohol consumption						
Light – moderate drinker	33 (43%)	27 (61%)	12 (46%)	3 (30%)	75	
Heavy drinker	15 (20%)	7 (16%)	8 (31%)	5 (50%)	35	
Non-drinker	8 (10%)	2 (5%)	3 (12%)	0 (0%)	13	
Ex-heavy drinker	2 (3%)	0 (0%)	0 (0%)	1 (10%)	3	***P* = 0.14 (NS)**
Alcohol status unknown	18 (24%)	8 (18%)	3 (11%)	1 (10%)	30	
Total	76	44	26	10	156	
Site of primary tumour						
Oral cavity	17 (22.4%)	5 (11%)	7 (27%)	1 (10%)	30	
Oropharynx	17 (22.4%)	31 (71%)	9 (35%)	2 (20%)	59	
Larynx	23 (30%)	2 (4%)	7 (27%)	6 (60%)	38	***P* < 0.001**
Other	19 (25%)	6 (14%)	3 (11%)	1 (10%)	29	
Total	76	44	26	10	156	
Grade of differentiation						
Well differentiated	1 (1%)	0 (0%)	0 (0%)	0 (0%)	1	
Moderately differentiated	32 (44%)	7 (18%)	11 (44%)	2 (25%)	52	
Poorly differentiated	33 (45%)	26 (67%)	12 (48%)	6 (75%)	77	***P* = 0.243 (NS)**
Moderate – poorly differentiated	7 (10%)	6 (15%)	2 (8%)	0 (0%)	15	
Total	73	39	25	8	145[a]	
Disease stage						
Stage I	13 (18%)	3 (7%)	4 (18%)	3 (30%)	23	
Stage II	12 (17%)	1 (2%)	3 (13%)	2 (20%)	18	
Stage III	12 (17%)	4 (9%)	6 (26)	3 (30%)	25	
Stage IVA & IVB	33 (45%)	32 (73%)	9 (39%)	1 (10%)	75	***P* = 0.02**
Unknown	2 (3%)	4 (9%)	1 (4%)	1 (10%)	8	
Total	72	44	23	10	149[a]	

Neck node status						
Negative	43 (59%)	5 (11%)	14 (54%)	8 (80%)	70	
Positive	30 (41%)	39 (89%)	12 (46%)	2 (20%)	83	***P* < 0.001**
Total	73	44	26	10	153[a]	
p16 status						
Positive	7 (9%)	32 (73%)	3 (11%)	2 (20%)	44	
Negative	69 (91%)	12 (27%)	23 (89%)	8(80%)	112	***P* < 0.001**
Total	76	44	26	10	156	
HPV status						
Positive	6 (9%)	29 (83%)	3 (12%)	1 (11%)	39[c]	
Negative	63 (91%)	6 (17%)	22 (88%)	8 (89%)	99	***P* < 0.001**
Total	69	35	25	9	138[b]	
Overall survival						
Alive	31 (41%)	27 (61%)	15 (58%)	6 (60%)	79	
Dead	45 (59%)	17 (39%)	11 (42%)	4 (40%)	77	***P* = 0.12 (NS)**
Total	76	44	26	10	156	
Recurrence						
Yes	18 (24%)	5 (11%)	1 (4%)	1 (10%)	25	
No	58 (76%)	39 (89%)	25 (96%)	9 (90%)	131	***P* = 0.067 (NS)**
Total	76	44	26	10	156	

a. **Patients with missing or unavailable data were excluded from analysis**
b. **Patients with 'Equivocal' HPV status were excluded from analysis**
c. **EBP50 staining could not be determined in one HPV-positive specimen**

Table 5.1: Social demographics and clinicopathological characteristics of patients based on EBP50 expression. Significant associations were noted between EBP50 expression and smoking history, site of primary tumour, stage of disease, metastasis to cervical lymph nodes, tumour p16 and HPV status. Weak/negligible cytoplasmic EBP50 expression demonstrated strong correlations to positive p16 and HPV status.

5.4.2 EBP50 expression and survival in patients with HNSCC

The relationship between EBP50 expression and overall survival in head and neck cancer patients was investigated. Kaplan Meier curves (Figure 5.8) revealed that patients with 'weak/negligible cytoplasmic' EBP50 expression had the highest overall survival while those with 'predominantly cytoplasmic' EBP50 expression had the worst survival (Median 49 months, 50% vs 68%; 5 years, 46% vs 60%, $P > 0.05$). Although overall survival rates were higher for the group with 'weak/negligible cytoplasmic' EBP50 expression, the smaller sample size of this cohort in relation to the original study cohort (156 vs 293) means that the significance of this correlation should be interpreted with caution (P =0.3).

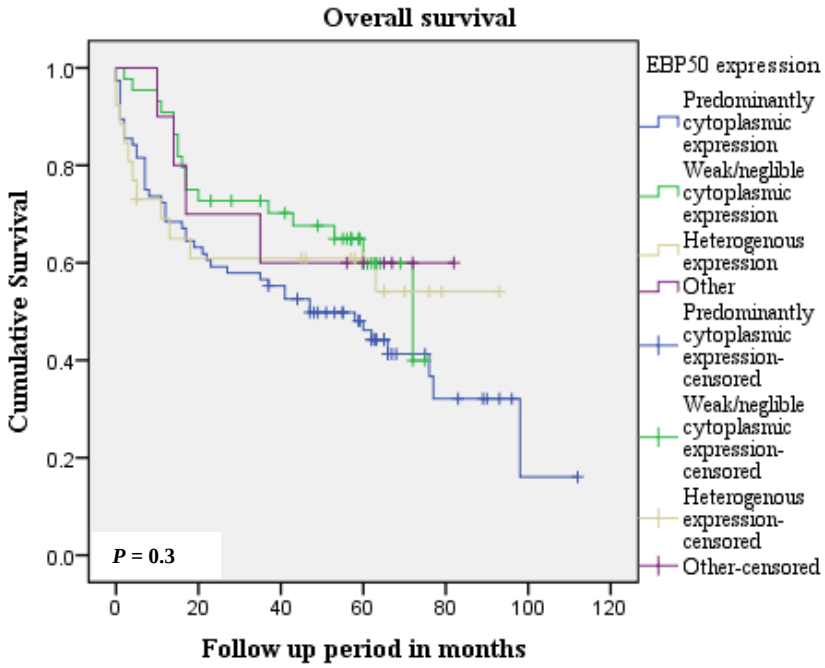

Figure 5.8: Kaplan-Meier plot for EBP50 expression and overall survival (OS). The association between EBP50 expression and overall survival in a cohort of 156 patients with HNSCC was analysed. Patients with weak/negligible cytoplasmic EBP50 had higher OS compared to those with 'predominantly cytoplasmic', 'heterogeneous' and 'other' expression patterns. Patients with 'predominantly cytoplasmic' expression tended to have the worst OS rates. The OS curves were analysed by Log Rank (Mantel Cox) test.

Similarly, Kaplan Meier curves for recurrence free survival (RFS) (Figure 5.9) revealed that patients with 'weak/negligible cytoplasmic' EBP50 expression had higher RFS rates compared to those with 'predominantly cytoplasmic' EBP50 expression (5 years, 64% vs 87%, P = 0.04). The impact of clinical and histological variables on RFS was

investigated through univariate survival analysis. Smoking status at diagnosis (*P* = 0.001), and EBP50 expression (*P* = 0.04) were found to be significantly associated with RFS. However, no significant independent associations were found following multivariate analysis.

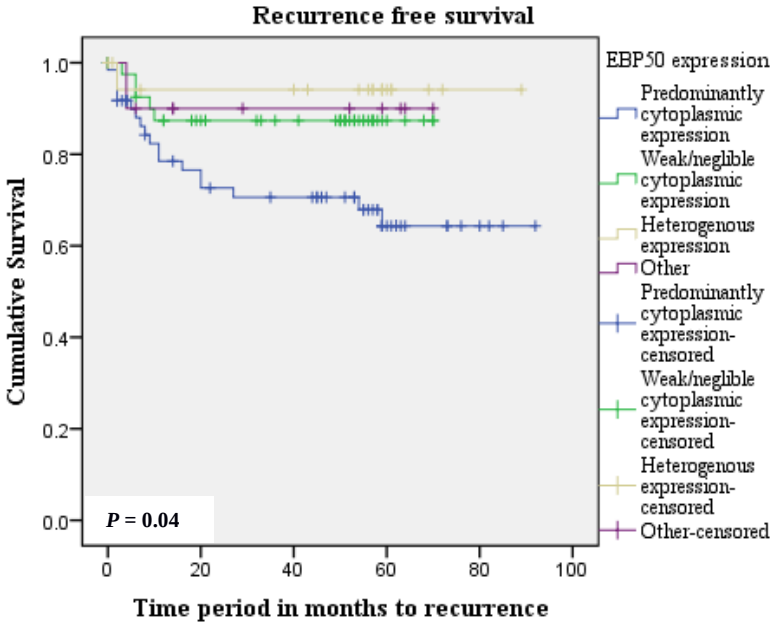

Figure 5.9: Kaplan-Meier plot for EBP50 expression and recurrence free survival (RFS). The association between EBP50 expression and recurrence free survival in a cohort of 156 patients with HNSCC was investigated. Patients with 'weak/negligible cytoplasmic' EBP50 expression were likely to be disease free longer than those with 'predominantly cytoplasmic' EBP50 expression. However, patients with 'heterogeneous' EBP50 expression appeared to have the highest recurrence free survival rates. RFS curves were analysed by Log Rank (Mantel Cox) test.

Interestingly, Kaplan Meier curves for disease specific survival (DSS) (Figure 5.10) revealed that patients with 'Heterogeneous' EBP50 expression had a lower risk of death from HNSCC compared to those with 'predominantly cytoplasmic' and 'weak/negligible cytoplasmic' EBP50 expression (2 years, 90% vs 83% and 79%, *P* = 0.331). Although patients with tumours exhibiting 'weak/negligible cytoplasmic' EBP50 expression appeared to have the highest risk of death from HNSCC at 2 years, they were more likely to have better survival rates compared to those with 'predominantly cytoplasmic' EBP50 expression in the long term. However, with a *P* value of 0.331 these results should not be considered statistically significant.

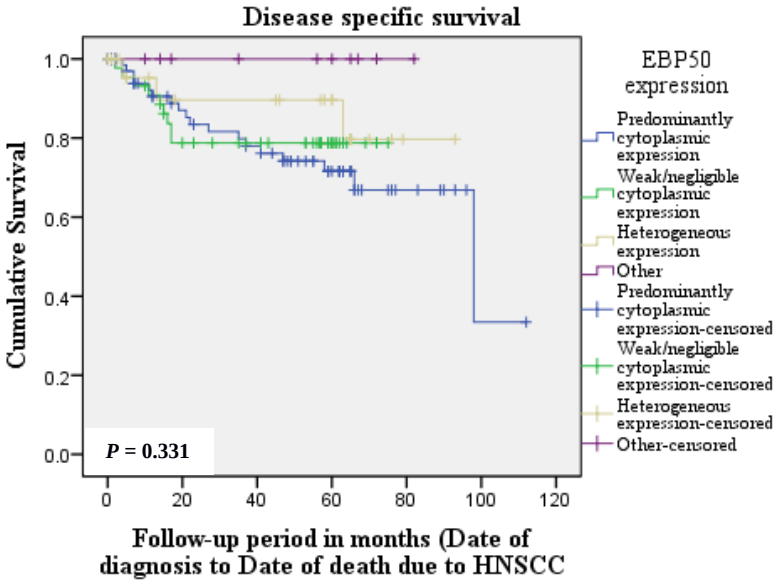

Figure 5.10: Kaplan-Meier plot for EBP50 expression and disease specific survival (DSS). The association between EBP50 expression and disease specific survival in a cohort of 156 patients with HNSCC was investigated. Patients with 'heterogeneous' EBP50 expression had a lower risk of death from HNSCC compared to those with 'predominantly cytoplasmic' and 'weak/negligible cytoplasmic' EBP50 expression. Patients with tumours exhibiting 'weak/negligible cytoplasmic' EBP50 expression appeared to have the worst prognosis until 3 years before showing a clear improvement over patients with 'predominantly cytoplasmic' EBP50 expressing tumours. DSS curves were analysed by Log Rank (Mantel Cox) test.

5.4.3 EBP50 as a potential marker of HPV infection in head and neck cancer

For the purpose of this study, a specimen was deemed HPV positive if it is p16 and HPV DNA positive. Therefore, one way to compare p16 and EBP50 expression as indicators of HPV infection is to test the relative strength of their dependence with HPV DNA status. Two independent Pearson's CHI Squared tests (χ^2) were performed on SPSS – one to test the dependence between EBP50 expression and HPV DNA status and the other to test dependence between p16 expression and HPV DNA status. Each test showed strong correlation between HPV DNA status and the indicator variables. The *P* values for both tests being identical ($P < 0.001$), the strength of goodness of fit of the variables was compared. The Likelihood Ratio for p16 status (87) was marginally higher than EBP50 expression (79.4) (Table 5.2).

HPV DNA Status (n = 156)	p16 overexpression		*P* value & Likelihood Ratio Value	EBP50 expression		*P* value & Likelihood Ratio Value
	Positive	**Negative**		**Weak/negligible**	**Other**	
Positive	39 (75%)	13 (25%)	***P* < 0.001 87**	38 (73%)	14 (9%)	***P* < 0.001 79.4**
Negative	5 (5%)	99 (95%)		6 (6%)	98 (63%)	

Table 5.2: EBP50 expression and p16 status correlation with HPV DNA status. Two independent Pearson's CHI Squared tests showed strong correlation between HPV DNA status and the indicator variables. The Likelihood Ratio for p16 status (87) was only marginally higher than EBP50 expression (79.4) indicating that Weak/negligible EBP50 expression may indeed be a novel marker of HPV-positive HNSCC.

5.5 Discussion

EBP50 was independently identified by Reczek and colleagues as a scaffolding protein which binds to the Ezrin-Radixin-Moesin (ERM) family of cytoskeletal proteins where they reciprocally stabilise each other to maintain a polarised state of epithelial cells [249]. Since then, numerous studies have reported on the potential role of EBP50 expression in carcinogenesis, including breast cancer [35], Schwanomma [87], hepatocellular carcinoma [276], colorectal [114] and gastric cancers [183]. EBP50 expression has been most extensively studied in breast cancer.

In this study, a preliminary investigation was undertaken to determine EBP50 expression by IHC in squamous cell carcinomas of the upper aerodigestive tract. This is the first study to investigate EBP50 expression and its implications on survival in head and neck cancer. In addition, this study also determined EBP50 expression in high-risk HPV positive tumours and attempted to establish its validity as a marker of HPV infection in HNSCC. High-risk HPV mediated degradation of EBP50 has been previously established by our lab and was also reported by Accardi *et al.* [2]. However, the impact of HPV positivity on EBP50 expression in head and neck cancer is a novel research topic.

Difficulties with background staining of tissue sections were encountered during optimisation of IHC protocol. A common problem in IHC analysis, background staining stems from several factors ranging from antigen-antibody reactions and detection methods to other general factors. Endogenous peroxidase activity, the attraction of

primary and secondary antibodies (Abs) to endogenous Fc receptors (FcRs) and tissue proteins are some of the contributing factors [117]. Tissue sections were incubated in varying concentrations of Hydrogen Peroxide (H_2O_2) to counter background staining possibly arising from endogenous enzymes. However, this step failed to completely eliminate it. Detection of unwanted background staining in sections incubated with secondary antibody only (i.e control without primary antibody) and those incubated with neutralised primary antibody indicated that the background may be related to interaction of the secondary antibody with endogenous FcRs or tissue proteins. While theories of antibodies reacting with endogeneous FcRs have been largely confounding [31], a more plausible explanation appears to be the increased hydrophobicity of tissue proteins resulting from cross-linking during fixation. Indeed, increased background staining as a result of hydrophobic and ionic interactions of tissue proteins is commonly seen in squamous epithelial tissue among others [117]. Further attempts to eliminate non-specific staining in the study cohort sections were discontinued as it did not interfere with the EBP50 signal.

As a preliminary step in this study, the distribution of EBP50 in normal tonsillar epithelium was investigated. In the suprabasal layers of the epithelium, prominent membranous EBP50 expression was observed while cytoplasmic EBP50 expression was almost negligible. However, a gradual increase in the strength of cytoplasmic staining was noted in the basal layers of the epithelium while plasma membranes of the epithelial cells in the basal layers were immunonegative for EBP50. EBP50 expression varies in different tissues. For example, in polarised epithelia such as liver, kidneys, trachea, small intestine, stomach and prostate, increased EBP50 expression with an intracellular distribution at apical luminal membranes of cells was reported [95]. In stratified squamous epithelium such as the skin, esophagus and tonsils, weak to moderate cytoplasmic EBP50 staining was observed tending toward stronger immunoreactivity in the deeper layers. While, eccrine glands in the skin have demonstrated cytoplasmic EBP50 with strong apical membranous immunoreactivity, adenexa showed weak diffuse cytoplasmic staining. Similarly in the serous acinar cells of the salivary glands, weak cytoplasmic EBP50 expression was accompanied by prominent linear luminous membranous staining [288]. Investigation of EBP50 expression in a cohort of 156 HNSCC specimens in this study uncovered 4 patterns of immunoreactivity. Predominantly cytoplasmic expression was seen in a majority of the samples followed by ‘weak/negligible cytoplasmic’ EBP50 expression, ‘heterogenous

expression' and other less common patterns. A noteworthy finding was the absence or lack of EBP50 expression in the plasma membranes of tumour cells. With the exception of a few samples with mixed membrane/cytoplasmic EBP50 expression, HNSCC specimens in this cohort were immunonegative for membranous EBP50. Examination of normal oral epithelium revealed that cells in the basal proliferative layer of the epithelium showed no immunoreactivity for EBP50 along the membranes. Therefore, a lack of membranous EBP50 expression in the tumour specimens may indicate that these tumours are comprised of a predominantly proliferative pool of epithelial cells. Alternatively, it may suggest a mis-localisation of the EBP50 protein from membrane to cytoplasm in squamous cell tumours of the head and neck. A similar EBP50 expression pattern was reported by Malfettone *et al*. following immunohistochemical analyses of colorectal tumour specimens. They found that while EBP50 was localised to the apical membranes of epithelial cells and in the cytoplasm of enterocytes of distant normal mucosa and mucosa adjacent to the tumour respectively, immunoreactivity in colorectal tumour samples was predominantly cytoplasmic [188], [186]. In another example, immunohistochemical analysis of normal and diseased breast tissue by Mangia *et al*., found that EBP50 expression was localised in the apical membranes of cells in normal breast tissue and in the cytoplasm of invasive breast carcinoma cells [187]. Interestingly, Malfettone *et al*. also reported nuclear expression of EBP50 [186]. However, no conclusive evidence of nuclear EBP50 immunoreactivity was found in this study.

The relationship between EBP50 expression patterns and clinicopathological characteristics of the cohort was analysed and significant associations were noted with smoking history at diagnosis, site of primary tumour, disease stage, lymph node status, p16 status and HPV status. Interestingly, 'predominantly cytoplasmic' and 'heterogeneous' EBP50 expression patterns were more likely to be seen in 'smokers' with tumours arising in non-oropharyngeal sites such as the oral cavity and larynx with no involvement of neck nodes. These patients were also more likely to present with a negative p16 and HPV status. On the other hand, 'weak/negligible cytoplasmic' EBP50 expression was noted in patients who were either 'non-smokers' or 'ex-smokers' at the time of diagnosis and had tumours arising in the oropharynx. Patients were more likely to present with positive neck nodes and late stage disease Furthermore, this expression pattern was strongly associated with a positive p16 and HPV status suggesting that tumours with 'weak/negligible cytoplasmic' EBP50 expression may have a different

pathology from those expressing ‘predominantly cytoplasmic EBP50. High-risk HPV E6 oncoprotein has the potential to interact with and mediate degradation of a subset of PDZ-containing cell polarity regulators including human Dlg (hDlg) and human Scribble (hScrib) [90], [214] and EBP50 [2]. Furthermore, HPV E6 targets specific cellular pools of cell polarity proteins [191], [145]. Consequently, it may be surmised that the reduced EBP50 expression evident in the group of predominantly oropharyngeal tumours in this cohort may be a result of high-risk HPV E6-mediated degradation of membrane pools of EBP50.

Paradoxically, no significant association was noted between EBP50 protein distribution and tumour differentiation in this study. EBP50 expression and tumour differentiation appears to be closely linked to the cellular compartment and the type of tumour in which it is expressed. For example, nuclear expression was reported to be strongly related to poor histological grade in colorectal tumours [188], whereas cytoplasmic EBP50 overexpression in breast cancer was not linked to tumour differentiation [235]. Interestingly, an investigation of the relationship between EBP50 expression as an independent variable and clinical outcomes of patients in the cohort showed that patients with ‘predominantly cytoplasmic’ EBP50 expression had the worst prognosis and reduced cytoplasmic EBP50 expression was associated with better OS and RFS. These results are very similar to survival data related to tumour HPV status in the cohort, although a statistically significant association of expression with OS was not observed. Previous reports on clinical outcomes and EBP50 expression are varied, seemingly linked to subcellular localisation of the protein [235], [183]. Paradiso *et al.* investigated the association of EBP50 expression in invasive breast carcinomas and clinical outcomes and found that cytoplasmic EBP50, high or low, did not correlate with OS or DFS [235]. Likewise, a study by Xiao-Guang *et al*. on EBP50 expression in gastric cancer found no correlation between overall survival rates and EBP50 expression [183]. Focusing on the two largest groups, patients with ‘predominantly cytoplasmic’ and ‘weak/negligible cytoplasmic’ EBP50 expressing tumours, and comparing their DSS survival rates, a marked improvement was observed for the latter group while the survival rate of the former group steadily declined over time. However, since the DSS analysis was not statistically significant, EBP50 expression may not be a valuable indicator of prognosis in patients with HNSCC.

5.6 Conclusion

The research undertaken in this chapter ascertained EBP50 expression in normal and diseased mucosal epithelium in the upper aerodigestive tract. EBP50 was expressed predominantly in the plasma membranes of cells in the suprabasal layers of normal oral epithelium becoming more cytoplasmic in the deeper layers. In a majority of study specimens, EBP50 expression was ‘predominantly cytoplasmic’ or ‘heterogeneous’ and the specimens were tumours involving the oral cavity, larynx, hypopharynx and pharynx. A smaller group of tumours mostly involving the oropharynx demonstrated reduced or negligible cytoplasmic EBP50 expression. Furthermore, this expression pattern strongly correlated with a positive HPV status. The preliminary findings in this chapter suggest that EBP50 expression is different in oropharyngeal and non-oropharyngeal tumours and this difference may be linked to its degradation by high-risk HPV.

Chapter 6

FINAL DISCUSSION

6.1 HPV in HNSCC and purpose of the study

Squamous cell carcinomas of the head and neck affect around 600,000 patients per year worldwide [83] and display wide aetiological, biological, phenotypical and clinical heterogeneity which makes them difficult to treat with a generalised treatment modality. HNSCC were typically associated with risk factors such as smoking and alcohol consumption, however, over the last decade, global incidence trends have witnessed a sharp rise in a subset of HNSCC that are aetiologically associated with high-risk HPV rather than the traditional risk factors [42]. Indeed, infection with high-risk HPV-16 is now recognised as an important risk factor for the development of OPSCC, particularly in the developed Western countries [326].

HPV-driven HNSCC represent a clinically distinct disease group compared to other HNSCC with affected patients tending to be younger males who are typically non-smokers [98]. Evidence suggests that oral HPV infection may be sexually transmitted [147] and that the increase in HPV-driven HNSCC may be in part due to changing sexual behaviour patterns [199]. Furthermore, since HPV-positive tumours respond better to chemotherapy and radiotherapy compared to HPV-negative tumours, patients have a better disease-free and overall survival [79] and also tend to have lower incidences of second primary tumours and a decreased risk of relapse [172]. This causal association between HNSCC and sexually transmitted high-risk HPV infection and improved clinical outcomes seen in patients with HPV-related HNSCC has important implications for the prevention, accurate diagnosis and treatment of HPV-associated HNSCC.

Currently, HPV testing in routine clinical practice is focused on accurate diagnosis of virus-induced cancer to inform clinical management. HPV infection is identified through DNA based detection methods such as PCR or ISH usually in combination with p16 IHC. Given the increasing global incidence of OPSCC, efforts should be made to understand the underlying pathogenesis better, identify cases early on in the disease process and, in the long run, prevent acquisition of oral HPV infection. Although over a hundred HPV subtypes have been identified, only over a dozen have been classified as

high-risk. Interestingly, head and neck cancer is almost always associated with high risk HPV type 16 which accounts for over 90% of the cases [282], [291] and, in addition, high-risk HPV does not appear to affect all mucosal head and neck anatomic sites equally as the vast majority of HPV-driven HNSCC arise from the mucosal lining of the oropharynx [282], [253], [167]. Given the close proximity of head and neck subsites and the challenges associated with attributing a site of origin for some large primary tumours, these statistics may be misleading. The reasons why most HNSCC arise as a result of infection with HPV-16 are not clear and viral predilection for the mucosal lining of the oropharynx needs further elucidation. Identification of additional molecular markers of HPV-driven HNSCC will shed light on the underlying pathogenesis of HPV-induced tumours of the head and neck.

Prevalence of HPV-associated HNSCC appears to vary with geographic location and time, with higher incidence rates in the US compared to Europe [119], [122], [49]. Apart from a recent report suggesting that OPSCC, possibly linked to HPV, is the fastest increasing cancer in men in Scotland [132], there are very few dedicated and comprehensive Scotland-specific studies which have reported on the incidence of HPV-positive HNSCC. In this direction, the research undertaken in this study has attempted to advance existing knowledge on the prevalence of HPV-driven HNSCC in Scotland. Currently, p16 IHC is the most commonly used surrogate marker of HPV infection but a growing body of research has expressed uncertainty regarding its efficacy as a reliable indicator of HPV-positive HNSCC [283], [141], [202]. While a novel single step detection method, the RNAscope, has been proposed by some groups [315], [263], there is scant research about other biomarkers of HPV. Data from our lab and a report by Accardi *et al.* [2] indicate that EBP50, an adaptor protein, is a novel target of the high-risk HPV E6 oncoprotein. This, and the existing dearth of information on the molecular markers of HPV-induced tumourigenesis in the head and neck, prompted an investigation of EBP50 and its expression in the study cohort. This study involved a retrospective analysis of archival FFPE specimens for a cohort of 293 HNSCC patients diagnosed and treated in NHS Tayside. The samples were tested for the presence of HPV DNA by PCR. These results, along with available p16 IHC results, were used to define HPV status of the cohort. A smaller subset of HPV-positive and negative samples were analysed for EBP50 expression patterns by IHC. The relationship between HPV status, EBP50 expression patterns and clinical characteristics of the cohort including survival outcomes was analysed.

6.2 Characteristics of the study cohort

As mentioned in the previous section, designating a site of origin for certain tumours in the upper aerodigestive track can be challenging because of the close proximity of head and neck subsites. Consequently, tumours arising in the base of the tongue or tonsil may sometimes be misclassified as originating in other head and neck subsites [135]. Therefore a relatively heterogeneous cohort, consisting of patients who were diagnosed and treated in a single centre, was selected for this study because it was more likely to reflect the true prevalence of HPV-related HNSCC in the region.

The crude incidence rates of HNSCC in the UK are strongly related to gender and age with a male : female ratio of 2:1 and an average patient age of 65 years [34]. More than half of the patients in this study cohort were males and the mean age of diagnosis was 64.5 years, which is in keeping with the general trends across the UK. These cohort characteristics are also comparable to those of a recent large scale prospective clinical cohort study of 5511 head and neck cancer patients recruited from various centres across the UK where the mean age of patients was reported to be 61 years and female patients comprised only 27% of the cohort [221]. Surprisingly, the distribution of tumours in this cohort, based on anatomic site of origin, was somewhat different from tumour categories reported in the Head and Neck 5000 study. While cancers of the larynx and oral cavity constituted the majority of cases in this cohort, oropharyngeal tumours constituted the largest category in the Head and Neck 5000 study followed by oral cavity and laryngeal tumours [221]. Given that the Head and Neck 5000 study is more recent (2011-2014) compared with our study (2006-2011), it is probably consistent with the trend of rising incidences in oropharyngeal cancers [34] [232], [128], [131]. Additionally, it is likely that the primary tumour site distribution trends noted in this cohort is a reflection of smoking and alcohol consumption habits of patients that configure it.

At diagnosis, more than a third of the patient population were smokers and nearly a fourth had a history of heavy drinking (current or ex-heavy drinker). These findings highlight negative health behaviour patterns in this population. Smoking/alcohol consumption and associated health outcomes such as long-term disease conditions and mortality often vary between countries, cities and regions [88]. Scotland is known to have a relatively poor health profile when compared to the rest of the UK and European countries and this, in turn, has been attributed to social deprivation [88], [36].

Interestingly, nearly half the study population was diagnosed with late stage disease, a finding consistent with other cohort studies [221]. An advanced disease stage at diagnosis has been associated with socioeconomic inequalities [229]. Although, an assessment of socio-economic backgrounds was not performed for this study, the Scottish Index of Multiple Deprivation 2013 reported Dundee city as having 30% of the share of the most deprived communities in Scotland.

HNSCC treatment often depends on the stage of disease at diagnosis and patients presenting with clinically advanced disease undergo multimodal treatment in the form of surgery, chemotherapy and/or radiotherapy [197]. Consequently, a large proportion of the patients in this cohort had undergone combined modality treatment in the form of chemoradiation, surgery with chemoradiation or surgery with radiotherapy and this is consistent with the general organ-preserving trends in treatment approaches noted in the literature [37], [85].

The 2-year OS and DSS rates for the study cohort were 62% and 82% respectively. Although the OS rate is comparable to that seen in the European population [107] it differs considerably from DSS rate. Patients with HNSCC often have comorbid conditions as a consequence of their smoking and drinking habits and have higher incidence of death from causes unrelated to head and neck cancer [259]. Smoking history at diagnosis, treatment modality and extracapsular spread were independent prognostic indicators for overall survival in this cohort and these observations are consistent with previous reports [63], [37], [121].

6.3 Incidence of high-risk HPV-related HNSCC in Tayside

One of the objectives of this study was to establish the prevalence of high-risk HPV and its impact on clinical outcomes in the study population. HPV testing for research purposes has been a continually evolving subject with considerable variability in the testing method of choice employed in cohort studies investigating viral incidence. In routine clinical practice in Scotland, HPV-related HNSCC is diagnosed either by p16 IHC alone or by employing a combination of p16 IHC and PCR (see Appendix A). The decision to investigate HPV status employing a combination of p16 and PCR in this cohort was influenced by the general trends in HPV testing prevailing in the literature at the time, its cost effectiveness as well as the fact that a p16 status was already available

for patients in the cohort.

Less than a quarter (14%) of the Tayside study population was found to be HPV-positive. The incidence rate reported in this study was much lower than the European average of 40% [1] and incidence rates of 70% reported in the US [54] but higher than that reported at a national level [4]. This variability may be attributable, in part, to geographic location [119]. For example, in a seminal study investigating the etiologic role of HPV in a large cohort of 253 patients with HNSCC in the US, Gillison *et al.* reported an incidence rate of 25% [101]. Our study was similar to that of Gillison *et al.* in sample size (293 vs 253) and type (heterogeneous) but a higher prevalence of HPV-associated HNSCC in North America may account for the higher incidence rate of 25% [148]. Other potential sources of variability include type of cancer sample analysed and the method of HPV detection employed in a study. For example, Wells *et al.* and Anderson *et al.* reported incidence rates of 45% and 10% respectively in Scottish cohorts. While Wells *et al.* investigated the etiological role of HPV in cohort of oropharyngeal tumours employing HPV DNA detection by PCR and p16 IHC, Anderson *et al.* employed PCR alone in a heterogeneous cohort of HNSCC patients [321], [4].

Not surprisingly, the vast majority of tumours in this cohort were high-risk HPV-16 positive and were tumours arising in the oropharynx - particularly in the tonsil. These results are consistent with other studies where heterogeneous cohorts were employed [1], [4]. HPV-positive tumours of oropharyngeal origin arise most commonly in the lingual and palatine tonsillar crypts [323]. It is hypothesised that the reticulated epithelium lining the tonsillar crypts provides an immune-privileged site by inhibiting virus-specific T cells and facilitating immune evasion at the time of initial HPV infection and subsequent virus-induced malignant transformation [323]. Furthermore, disruptions in the reticulated epithelium leave the basement membrane exposed to viral particle deposition without the need for mechanical abrasion of the mucosa, making the tonsils more susceptible to HPV infection and subsequent malignant transformation [323]. High-risk HPV was also detected in tumours arising in the oral cavity, the pharynx and the nasal cavity. The prevalence of HPV in non-oropharyngeal head and neck subsites has been somewhat dichotomous. For example, a prevalence of 16% of high-risk HPV was reported in a recent cohort study of Taiwanese patients with squamous cell carcinomas of the oral cavity [163]. In another heterogeneous cohort

study Gillison *et al.* reported the presence of HPV DNA in the oral cavity (16%), larynx (26%) and the hypopharynx (3%) [101]. In a systematic review, Kreimer *et al*. [148] reported that 23.5% of oral cavity tumours and 24% of laryngeal tumours were HPV DNA positive, findings which are similar that reported by Mehanna *et al*. [194]. It is important to note here that the findings reported by these studies were based predominantly on HPV DNA detection by PCR and are considerably higher than the incidence rates noted in non-oropharyngeal sites in this study (7/293; 2.3%). Contrarily, Lopes *et al.* were able to demonstrate that the prevalence of high-risk HPV in squamous cell carcinomas of the oral cavity was very low by employing a combination of consensus and quantitative PCR and ISH, [178]. Given the tendency of PCR based methods to be non-specific when used alone, the higher incidence rates seen in the literature may be misleading. The findings of low HPV prevalence in non-oropharyngeal sites reported this study concur with that of Lopes *et al*. [178] and provide additional evidence that the oropharynx has a higher predilection for transformation by oncogenic HPV. However, the etiologic role of high-risk HPV in non-oropharyngeal sites remains unclear and highlights the need for additional large scale epidemiological studies to uncover any significant associations.

Paradoxically, only 24 out of 40 HPV positive samples in this cohort also tested positive for high-risk HPV by DNA ISH. ISH differs from PCR and p16 IHC in that it allows for detection of HPV DNA within the nuclei of infected tumour cells. Additionally, the physical state of the virus as indicated by punctate staining for integrated virus and diffuse staining for episomal virus is also evident with ISH [310]. However, a major drawback of the ISH is its lack of sensitivity as it is only able to detect HPV when there are around 10 copies of virus per cell [310]. Despite, the high specificity of this method, estimated false negative rates of 13-41% have been reported in HNSCC owing to its low sensitivity and a tendency for probe cross hybridisation [308], [40]. While a lack of concordance between DNA ISH and other routinely used HPV testing methods has been reported by previous studies [282], [269], the reason for discrepancies observed in this study is unclear and may be due to reduced copy numbers/positive tumour cells in the specimens. However, a comment on the feasibility of DNA ISH for risk stratification in routine clinical practice is beyond the scope of this thesis.

Overall survival and recurrence free survival was significantly improved for HPV-positive patients in this cohort. Furthermore, tumour HPV status was an independent

prognostic factor for overall survival. The 2-year OS rate for HPV-negative patients was 57% compared to 77% for HPV-positive patients. These results are comparable to a study by Evans *et al.* in a Welsh cohort where 3-year survival was reported to be 32.2% for HPV-negative patients and 82.6% for HPV-positive patients [75]. However, the study by Evans *et al.* [75] comprised patients with oropharyngeal tumours only and it is relevant to reiterate, in this context, the scope and depth of the study in this thesis in relation to other studies undertaken. Several groups have reported on favourable prognosis conferred by HPV-positivity in HNSCC [79], [174], [173], but some others have reported contradictory findings which showed no association between HPV-positivity and patient prognosis [141], [71]. One hypothesis to explain these conflicting results is that most studies reporting improved clinical outcomes in HPV- positive cancers were conducted on predominantly oropharyngeal tumour cohorts. Overall survival in this study was not influenced by the heterogeneity of the cohort. On the other hand, no significant associations were found between DSS and HPV status in this cohort. The findings of studies reporting on the association between HPV status and cause specific survival are varied. For example, Wells *et al.* [321] and Dahlgren *et al.* [57] found a significant association between HPV status and cause specific survival. However, both studies were carried out on a cohort of oropharyngeal/tonsillar squamous cell carcinomas and fresh frozen biopsy specimens were employed in the study by Dahlgren *et al.* [57]. On the other hand, Ritchie *et al.* [254] found no significant association between tumour HPV status and DSS, a finding concurrent with this study. The differences in cause specific survival between this study and others may be attributed to differences in cohort type and size and in percentages of deaths due to cancer. Additionally, disease specific survival curves can often be misleading as they do not include death caused by disease related factors such as treatment [252].

6.4 EBP50 - a novel marker of HPV-associated HNSCC

The second objective of this study was to evaluate EBP50, a cytoskeletal adaptor protein targeted by the high-risk HPV, as a novel diagnostic marker for HPV-related HNSCC. HPV and its association with HNSCC is a continually evolving field of research with numerous studies investigating diagnostic testing and treatment modalities for patients. However, very little research has been undertaken to uncover the molecular events that lead to HPV-induced tumorigenesis. A widely accepted hypothesis is that the virus infects the proliferating basal layer of epithelial surfaces at a site of injury. By

subsequent internalisation, evasion of host immune response and replication, the virus propagates persistent infection [202]. Viral integration into the host genome is an important step resulting in the overexpression of viral oncoproteins E6 and E7. Overexpression of E6 and E7 leads to disruption of the p53 and pRB pathways, among others, resulting in cell proliferation and genomic instability [161], [25]. Overexpression of p16, a surrogate marker of HPV infection, is one such consequence of the E7 oncoprotein mediated disruption of the pRB pathway. However, p16 overexpression has also been observed in the absence of HPV positivity, in both oropharyngeal and non-oropharyngeal sites in the head and neck and has been postulated to be due to genetic mutations such as loss of Rb or amplification of Cyclin D1 [328]. Additionally, the oncogenic activity of E6 has been attributed to its ability to bind to and degrade PDZ domain containing cell polarity proteins including EBP50 [204], [2].

In this study, the influence of high-risk HPV on EBP50 was investigated by immunohistochemical analysis of a cohort of HPV-positive and negative HNSCC patients. Four groups of EBP50 expression patterns were identified; 'predominantly cytoplasmic', 'weak/negligible cytoplasmic', 'heterogeneous' and 'other' less common patterns. The majority of tumour samples demonstrated a 'predominantly cytoplasmic' EBP50 expression pattern. Interestingly, membranous EBP50 expression was absent in nearly all the samples in the cohort except for a few with 'heterogeneous' and 'other' staining patterns. This is in stark contrast to membranous EBP50 immunoreactivity observed within the supra-basal layers of the epithelium in a normal tonsillar specimen in this study and also reported in other tissues [95]. While the role of EBP50 has been studied in breast cancer [235], Schwanomma [87], hepatocellular carcinoma [276], colorectal and gastric cancers [186], [183], this is the first study to report on its expression in HNSCC. A 'predominantly cytoplasmic' EBP50 expression accompanied by a complete absence of membranous immunoreactivity has been previously reported in breast and colorectal carcinoma [95], [186]. Gerogescu *et al*. noted that EBP50 is either absent or 'overexpressed in an altered intracellular distribution' in tumours and proposed that EBP50 may function as a tumour suppressor when it is localised at the plasma membrane and as an oncogenic protein when it is localised in the cytoplasm [95]. The absence of membrane EBP50 and its expression in the cytoplasm observed in a large number of HNSCC samples in this study may represent an altered protein distribution and appears to suggest a tumour suppressor role for EBP50.

EBP50 proteins possess two PDZ binding domains and a C-terminal binding domain through which they form protein-protein complexes at the interface of the plasma membrane and cytoplasm [249]. Formations of such protein-protein complexes allow them to exercise anchoring functions. For instance, EBP50 associates with β-catenin forming an important component of the E-cadherin-β-catenin protein complex for cortical stabilisation in epithelial cells [276]. Similarly, it binds to Epidermal Growth Factor (EGFR), a transmembrane protein implicated in carcinogenesis [329]. Disruption of EBP50 protein complexes could trigger a sequence of events leading to a malignant phenotype. In a study investigating EBP50-EGFR interplay, Claperon *et al.*, reported that a loss of EBP50 at the plasma membrane and cytoplasmic overexpression in tumour cells may contribute to biliary carcinogenesis by EGFR activation [48]. The authors were able to demonstrate that depletion of EBP50 in biliary carcinoma cells increased the activity of EGFR at the cell surface and this enhanced EGFR activity triggered a disruption in adherens junctions by inducing internalisation of E-cadherin and β-catenin [48]. Indeed, overexpression of EGFR and its ligand TGFα is commonly seen in HNSCC [105]. Thus, it can be hypothesised that an aberrant activity of the EGFR signalling pathway brought about by ectopic localisation of the EBP50 protein and subsequent loss of its tumour suppressor function may, in part, contribute to the formation of squamous cell carcinomas of the head and neck.

An alternative mechanism by which EBP50 in squamous epithelial cells may contribute to tumour formation is by directly influencing the β-catenin/Wnt signalling pathway as seen in colorectal cancer [114]. β-catenin is stabilised at cell-cell junctions through its interaction with EBP50 [146]. *In vitro* studies by Hayashi *et al.*, found that depletion of EBP50 from plasma membranes of polarised intestinal epithelial cells induced in Epithelial Mesenchymal Transition (EMT), nuclear translocation of β-catenin with elevation of Wnt/ β-catenin transcriptional targets, and increased cell migration and invasion. Normal cell morphology and reduced motility were restored only by re-expression of the protein specifically at the plasma membrane, reinforcing the tumour suppressor function of EBP50 at plasma membranes of epithelial cells [114].

Interestingly, ‘predominantly cytoplasmic’ EBP50 expression patterns were more likely to be seen in ‘smokers’ with tumours arising in non-oropharyngeal sites such as the oral cavity and larynx with no involvement of neck nodes. These patients were also more likely to present with a negative p16 and HPV status. On the other hand, a smaller

proportion of the study samples demonstrated a ‘weak/negligible’ cytoplasmic staining and patients with tumours exhibiting reduced cytoplasmic EBP50 were ‘non-smokers’ or ‘ex-smokers’ at the time of diagnosis and had tumours arising predominantly in the oropharynx. These patients were also more likely to present with positive neck nodes and late stage disease and were positive for p16 and HPV. Thus, each EBP50 staining pattern correlates with a distinct patient profile very similar to characteristics of patients with HPV-positive and negative tumours.

A sustained high-level expression of HPV oncoproteins E6 and E7 is vital to the development and maintenance of virally induced malignant phenotype. The high-risk HPV E6 oncoprotein has a PDZ binding motif (PBM) in its carboxyl terminus, a feature absent in low-risk benign HPVs, which allows it to interact with PDZ domain containing cell polarity proteins [10]. In addition to contributing to E6-mediated malignant transformation of tissues, E6-PDZ domain protein interactions play a crucial role in the viral life cycle. For example, viral interaction with hScrib has been shown to stabilise E6 in the early stages of the viral life cycle by increasing the levels of E6 protein [226]. Such PBM-dependent stabilising interactions with E6 have also been suggested for other PDZ domain containing proteins such as hDlg1 and MAGI-1 [213]. Such a stabilising role for EBP50 is yet to be uncovered. It is evident, however, that HPV-16 E6 interaction with EBP50 may support malignant transformation of epithelial tissue by the virus. Accardi *et al.* were able to demonstrate that HPV-16 E6 binds to and induces degradation of the EBP50 protein *in vitro* and is assisted by the E7 oncoprotein [2]. In addition to their *in vitro* data, the authors were also able to show that EBP50 levels were very low in SiHa and CaSki cell lines, both of which are HPV-16-positive cervical cancer derived cell lines. Based on this evidence, low or negligible levels of EBP50 observed in a proportion of predominantly oropharyngeal HPV-16-positive tumours in this cohort is suggestive of a virus induced degradation of the cell polarity protein. Cavatorta *et al.* reported similar findings of low or absent expression of Dlg, a PDZ substrate of high-risk HPV, in invasive cervical carcinoma specimens where low levels of the protein correlated with the presence of high-risk HPV [38]. Furthermore there are reports of E6 targeting specific cellular pools of substrate proteins. For example, HPV E6 has been shown to specifically target nuclear pools of hDlg and nuclear and membrane bound forms of MAGI-1 [145]. Based on findings of this study, it may be hypothesised that the low/negligible EBP50 protein levels seen in HPV-positive tumour cells may be a result of E6 targeted degradation of membrane bound

pools of EBP50. The E6 oncoprotein has been shown to inactivate PTEN through PDZ domain proteins, leading to increased pAkt activation and subsequent increased cell proliferation [45]. EBP50 has been shown, *in vitro,* to facilitate the formation of a ternary complex of the PTEN tumour suppressor protein with PDGFR at the plasma membrane of mouse embryonic fibroblasts thus keeping in check the activation of PI3K pathway in response to PDGF stimulation [297]. It is possible that malignant transformation in HPV-infected tissues may in part be a result of E6-mediated degradation of EBP50 and subsequent disruption of the PTEN-PDGFR complex leading to a prolonged activation of the Akt/PI3K signalling pathway. Indeed, Accardi *et al.* were able to demonstrate that HPV E6-mediated degradation of EBP50 induced activation of the Akt/PI3K signalling pathway [2].

A few HPV-positive specimens in this study did not demonstrate 'weak/negligible cytoplasmic' EBP50. E6 phosphorylation by Protein Kinase A (PKA) inhibits its ability to bind to and degrade PDZ domain proteins [152] and it is possible that EBP50 degradation in these tumour samples was inhibited by PKA phosphorylation. A smaller proportion of study samples demonstrated a mixed or 'heterogeneous' EBP50 expression pattern in which the protein could be detected in the plasma membranes of a few tumour cells while it was either absent or weak in the others. Likewise, a mixed cytoplasmic expression was also noted wherein a few specimens demonstrated cytoplasmic EBP50 immunoreactivity ranged from clear positive to weak or negligible. The clinicopathological characteristics of patients with tumours exhibiting a mixed expression pattern were similar to those with 'predominantly cytoplasmic' expression. It may be surmised that tumour cells demonstrating mixed EBP50 expression represent various stages of loss of tissue architecture and tumour invasiveness. Although 'weak/negligible cytoplasmic' EBP50 expression was associated with improved clinical outcomes in a univariate analysis, EBP50 expression was not an independent prognostic factor in multivariate analysis suggesting that other factors may compete or interact with EBP50 expression to influence survival [235].

The preliminary findings on EBP50 expression in HNSCC reported in this study support the hypothesis that downregulation of EBP50 may contribute to HPV-mediated malignant transformation of epithelial tissues in the head and neck region making EBP50 a potential marker for HPV-related HNSCC. Furthermore, it may be surmised that the loss of tumour suppressor function of EBP50 either by aberrant localisation or

downregulation/degradation by high-risk HPV may be implicated in the molecular pathogenesis of HNSCC, albeit through different signalling pathways.

6.5 Strengths of the study

This study included a large, well-characterised cohort of all HNSCC cases from a geographically defined population diagnosed over a six-year period and treated at a single NHS centre. The sample size of 293 patients is much larger than that of other UK- wide cohort studies investigating the prevalence of HPV in HNSCC. Furthermore, this cohort not only includes tumours arising in the oropharynx but also from other head and neck subsites. Patient case notes were reviewed to record missing clinical data and to update survival data. This ensured that the existing database was current and complete. The use of official death certificate records (General Register Office) ensured that the key clinical outcome of disease specific survival was ascertained with accurate data. Meticulous selection of tumour specimens based on the presence of a substantial amount of tumour tissue and its suitability for downstream experiments ensured that the study cohort is robust. Scoring of ISH results and EBP50 IHC staining patterns was undertaken by two independent observers and EBP50 staining in 10% of the study samples was additionally scored by a third observer ensuring that the interpretation of test results is free of bias.

6.6 Limitations of the study

This was a retrospective study and data pertaining to certain pathological features such as ‘field change’, ‘perineural invasion’ and ‘lymphovascular invasion’ was missing. This was also due to inclusion of cases into the study where surgery was not a treatment modality and available specimens were diagnostic biopsies. Clinical data pertaining to smoking and alcohol was based on information provided by the patients at the time of diagnosis. Smoking history was not available in pack-years. At the end of the study mean length of follow-up was 39 months and only three quarters of the study population was followed up for 5 years. Cause of death data was obtained from the GRO and may be subject to errors as is the case with many national registration systems. Thus by relying on information from GRO death certificates, the number of head and neck cancer deaths may have been overestimated. Analysis of EBP50 expression in normal mucosa of the upperaerodigestive tract was restricted to a single specimen owing to the unavailability of normal specimens.

6.7 Significance of the study

The diagnosis, treatment and prevention of HPV-related HNSCC is a complex problem and there is a critical need for additional diagnostic and prognostic biomarkers. Current literature on the prevalence of high-risk HPV in the Scottish population is limited by small study samples with a focus on specific subsites. Furthermore, ongoing research is largely directed toward treatment of HPV-positive tumours and there is relatively little information on the underlying molecular pathogenesis of HPV induced tumorigenesis. This study is the largest retrospective analysis of HPV incidence in a heterogeneous Scottish cohort. It is also the first study to report on EBP50 expression in HNSCC. The results obtained from HPV DNA analysis by PCR of tumour samples combined with p16 status has contributed to existing knowledge on the global incidence of HPV-associated HNSCC. It has also highlighted potential negative health behaviours in the population and the need for patient education and motivation. Furthermore, the findings on EBP50 expression provide preliminary but strong evidence in support of the diagnostic value of EBP50 as a marker of HPV-related HNSCC.

Chapter 7

CONCLUSION

HPV-associated HNSCC represent a distinct disease entity with unique clinical and molecular characteristics. There is considerable variability in the reported incidence rates of HPV-related HNSCC worldwide depending on the geographic area and type of HPV detection assay complicated further by challenges associated with assigning a 'site of origin' for larger tumours given the close proximity of head and neck subsites [135]. The base of the tongue and palatine tonsils are the most commonly affected head and neck anatomic subsites and also where survival advantage of HPV-positive tumour status has been extensively reported [79], [99], [321], [295]. Occasionally, other subsites such as the larynx, oral cavity and hypopharynx are affected. However, the prognostic significance of HPV positivity in these subsites is debatable. Recently, OPSCC was reported as the fastest increasing cancer in men in Scotland and it was suggested to be linked to infection with high-risk HPV [132]. Very few studies have investigated the incidence of HPV in HNSCC across the UK, fewer still in Scotland. Most of them are limited by small study samples and a tendency to focus on specific head and neck subsites. Furthermore, there is very little research on molecular pathogenesis and biomarkers of HPV-driven tumorigenesis as most studies focus on virus detection and treatment of HPV-related HNSCC.

This retrospective study set out to determine the prevalence of high-risk HPV infection in a heterogeneous cohort of HNSCC patients diagnosed and treated in a single NHS treatment centre. It also sought to explore expression patterns of the cytoskeletal adaptor protein EBP50, a target of high-risk HPV E6 oncoprotein, in a subset of HNSCC samples.

The specific aims of this study were

1) To determine the prevalence of high-risk HPV and its impact on clinical outcomes in a sample population of HNSCC patients in Tayside, Scotland.

2) To evaluate EBP50, a cytoskeletal adaptor protein targeted by the high-risk HPV, as a novel marker for HPV-related HNSCC.

The main experimental findings of this study are chapter specific and are summarised within Chapters 4 (Prevalence of Human Papillomavirus related HNSCC in a Tayside

cohort) and 5 (Investigation of EBP50 as a potential novel marker of HPV associated HNSCC). The main findings with respect to the study aims will be summarised in the following sections.

7.1 Determination of the prevalence of high-risk HPV and its impact on clinical outcomes in the study population

7.1.1 Prevalence of HPV in the sample population

The p16 status of all the study samples was already available. HPV DNA status of all the study samples was determined by PCR using consensus and type specific primers. High-risk HPV infection was detected in less than a quarter of the cohort. The majority of the cohort was HPV negative (80%) and a small proportion (6%) was 'equivocal'. All the study HPV-positive samples, except one, were infected with high-risk HPV type 16.

7.1.2 Influence of HPV on patient demographics and tumour histopathology

The social demographics and clinical characteristics of patients with HPV-positive tumours were different from those with HPV-negative tumours. These patients were more likely to belong to a younger age group of 58 years or under and were not smokers at the time of diagnosis. The majority of the HPV-positive tumours were of oropharyngeal origin, poorly differentiated and did not demonstrate features of tumour aggression such as the presence of extracapsular spread and cohesive invasive tumour front.

7.1.3 Influence of HPV on all cause, disease specific and disease free survival

As expected, a greater proportion of survivors were seen in the HPV-positive group compared to the HPV-negative group. Patients with HPV-positive tumours demonstrated an improved overall and recurrence free survival. However, HPV-positivity was not a prognostic indicator for disease specific survival.

7.2 Evaluation of EBP50 as a novel marker for HPV-related HNSCC

7.2.1 EBP50 expression in normal oral mucosa and primary HNSCC

EBP50 expression in normal tonsillar mucosa and a subset of HPV-positive and negative tumour samples was analysed by immunohistochemistry employing a

polyclonal anti-EBP50 antibody. In normal oral mucosa, EBP50 expression is predominantly membranous in suprabasal layers of the epithelium with a gradual shift in distribution from membranous to predominantly cytoplasmic in the basal layers of the epithelium. On the other hand, absence of EBP50 expression in the plasma membranes of tumour cells was a recurring pattern in a majority of the tumour samples. Furthermore, EBP50 was predominantly expressed in the cytoplasm of nearly half of the tumour samples. In a smaller proportion of the samples cytoplasmic EBP50 was either absent or very weak. Mixed EBP50 immunoreactivity was observed in a very small proportion of tumour samples.

7.2.2 EBP50 expression in relation to clinicopathological characteristics of the study cohort

EBP50 expression was significantly associated with smoking status at the time of diagnosis, site of primary tumour, disease stage, neck node status, HPV and p16 status. Weak or negligible cytoplasmic EBP50 expression was observed largely in patients with tumours of the oropharynx presenting with advanced disease, no history of smoking at the time of diagnosis, positive p16 and HPV status. In contrast, cytoplasmic EBP50 expression was observed primarily in patients with a smoking history, non-oropharyngeal tumours, negative p16 and HPV status. EBP50 expression showed a strong correlation, only marginally lower than p16 overexpression, with HPV DNA status.

7.2.3 EBP50 expression in relation to clinical outcomes of the study cohort

Although patients with weak/negligible cytoplasmic tumour EBP50 expression showed improved all cause, recurrence free and disease specific survival, no significant associations were found between clinical outcomes and EBP50 staining.

7.3 Implications of the study findings

Data and statistics provided by population based studies are essential for determination of the true prevalence of a disease. HPV in HNSCC in the Scottish population has been previously reported by Anderson *et al*. [4] and LAR Wells *et al*. [321]. While the prevalence reported in this study contributes to existing knowledge on HPV incidence in head and neck cancer in Scotland, it varies from reported results from other regions. This may be attributed to sample selection and size and primers used in HPV DNA

detection. The clinical characteristics of the sample population and histopathological traits based on tumour HPV status reported in this study are concurrent with current literature [155]. HPV positivity correlated with improved overall and recurrence free survival, but not with cause-specific survival. These results are, to a small degree, incongruous with those reported by LAR Wells *et al*. [321] and may be attributed to the heterogeneous nature of the sample population in this study. Indeed, improved survival is often reported in oropharyngeal tumour cohorts [272], [5] while mixed or non-oropharyngeal cohorts have shown no difference in survival between HPV-positive and HPV-negative patients diagnosed with head and neck cancer [240].

The novel research undertaken in this study has shown that EBP50 is differentially expressed in HNSCC depending on tumour HPV status. It certainly contributes to recent evidence that regulation of EBP50 by HPV has a crucial role in HPV-associated carcinogenesis [2]. The 'predominantly cytoplasmic' staining pattern observed in nearly half of the study cohort has been previously reported in colorectal and breast cancer [114], [187]. These findings are suggestive of a shift in subcellular localisation of the EBP50 protein from membrane to cytoplasm in squamous cell tumours of the head and neck and reinforce a tumour suppressor role for EBP50. Weak/ negligible cytoplasmic EBP50 expression seen in a small proportion of tumours that originated predominantly in the oropharynx and were HPV-positive is suggestive of a virus induced degradation of the cell polarity protein. These findings parallel those of Cavatorta *et al.* on the influence of high-risk HPV on hDlg in cervical cancer specimens [38]. Clinical and histological characteristics of the two dominant EBP50 expression patterns are similar to those reported for HPV-negative and positive head and neck tumours and provide further evidence supporting a two distinct disease processes.

EBP50 expression was not an independent prognostic indicator in HNSCC. Although improved survival rates were noted for reduced cytoplasmic EBP50 expressing tumours, only recurrence free survival was significant. These results, although not statistically significant, are very similar to survival data related to tumour HPV status in the cohort. Previous reports on clinical outcomes and EBP50 expression are varied, seemingly linked to subcellular localisation of the protein [183], [235]. It is possible that the survival rates noted in this study are merely reflective of the HPV status of the tumours and its influence on survival. Furthermore, other factors may compete or interact with EBP50 expression to influence survival. Therefore, the findings of this study also

provide evidence that unlike p16, EBP50 expression is not an independent prognostic indicator in HNSCC.

The findings of this study support current consensus that HPV-positive HNSCC are different from HPV-negative HNSCC in its clinical features, tumour histopathology and molecular traits. The prevalence of high-risk HPV in this Scottish cohort was relatively low compared to that of other countries. Findings on social demographics and clinical characteristics have highlighted negative health behaviours in this population indicating a need for patient education for preventive healthcare and screening programmes that are accessible to all communities. Preliminary findings on EBP50 expression in this cohort indicate that it may be a valuable marker for HPV- positive HNSCC. It also suggests that loss of EBP50 may support tumour progression through different signalling pathways in HPV-positive and negative head and neck cancer and its modulation may be a potential therapeutic strategy in improving clinical outcomes in these patients.

7.4 Future direction

The variability in incidence rates across studies reiterates the need for uniform and standardised HPV testing protocols. Current literature does not provide conclusive evidence on the etiologic role of HPV in non-oropharyngeal head and subsites and additional large-scale cohort studies are needed to elucidate significant associations, if any, and related impact on patient prognosis. Additionally, given the rising incidence of oropharyngeal cancers in Scotland, a large prospective cohort study of oropharyngeal patients would establish current HPV prevalence in the Scottish population and whether incidence rates have changed over time. Further studies on EBP50 expression in normal, dysplastic, carcinoma *in situ* specimens will be helpful in determining whether loss of membranous EBP50 and its aberrant subcellular localisation is an early or late stage marker in head and neck tumour progression.

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APPENDIX A

Practice trends in HPV testing for HNSCC

Presented in this section are excerpts from a Scotland-wide survey on practice trends in HPV testing for head and neck squamous cell carcinomas which was undertaken concurrently with the research in this thesis.

Oral pathologists/general pathologists specialising in head and neck pathology from each of the Scottish NHS boards were invited to take part in a survey on routine diagnostic HPV testing for HNSCC in their local NHS centres. A participation invite with the survey link was circulated among the specialists via the Scottish pathology network (SPaN).

A. Diagnostic testing for high-risk HPV-related HNSCC

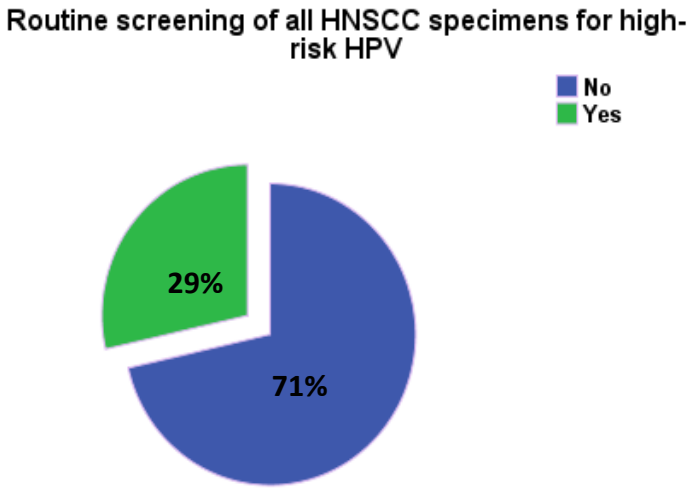

Figure A: Routine screening of all HNSCC specimens for high-risk HPV was reported by 29% of the respondents. The vast majority, however, said they tested for HPV only when the specimens were from a specific subsite like the oropharynx or when they received a special clinical request for HPV status. Age of the patient and discussions at multidisciplinary team meetings were also some other circumstances under which diagnostic HPV investigations were carried out.

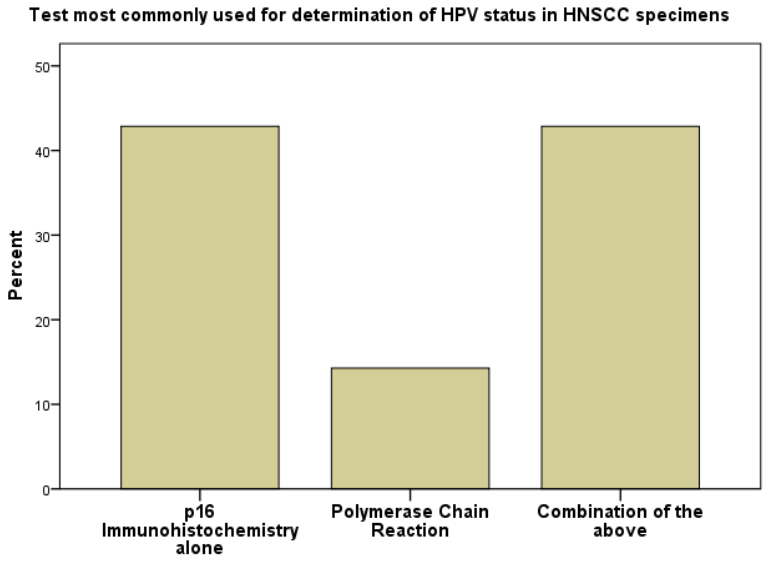

Figure B: The p16 immunohistochemistry was the most common single assay used in practice. An equal proportion of respondents said they employed p16 as first line assay followed by PCR. PCR as a lone assay for determining tumour HPV status was employed by one NHS board.

B. Interpretation of p16 immunohistochemistry results and scoring methods

A general lack of consistency was observed in the interpretation of p16 IHC results across the NHS boards. Specimens were scored: as positive for HPV if 70% or more of the tumour cells showed nuclear and cytoplasmic staining (33%), as positive, equivocal or negative for HPV based on the presence or absence of staining (33%), positive for HPV if tumour cells showed strong diffuse nuclear staining (17%).

C. Variability in practice trends among specialists of the same NHS boards

	Board A			
Survey Question	Participant 1	Participant 2	Participant 3	Participant 4
Which test/tests do you most commonly use for determination of HPV status in a HNSCC specimen?	In Situ Hybridization	p16 followed by PCR	p16 IHC	p16 IHC

	Board B	
Survey Question	Participant 1	Participant 2
Please provide details about the scoring method for p16 overexpression	Negative, Equivocal or Positive	50% or more tumour cells showing nuclear and cytoplasmic staining

Figure C: Variability in testing practices and reporting of results was observed within individual health boards. For example, out of four respondents from Board A, two respondents employed p16 IHC as a lone test for determination of tumour HPV status while the others used ISH and p16 along with PCR. In Board B, the scoring protocol used to report p16 IHC results by two respondents were different.

APPENDIX B

Results of HPV genotyping

Table A: Result of HPV genotyping

Specimen ID	Beta Globin	HPV L1	HPV E1	HPV Status_based on genotyping for L1 or E1 gene	Type specific PCR
1	Positive	Negative	Negative	Negative	NA [a]
2	Positive	Negative	Weak positive	Weak positive	HPV 18
4	Positive	Negative	Negative	Negative	NA
5	Positive	Negative	Negative	Negative	NA
6	Positive	Negative	Negative	Negative	NA
7	Positive	Negative	Negative	Negative	NA
8	Positive	Negative	Negative	Negative	NA
9	Positive	Negative	Negative	Negative	NA
10	Positive	Negative	Negative	Negative	NA
11	Positive	Negative	Negative	Negative	NA
12	Positive	Negative	Negative	Negative	NA
14	Positive	Negative	Negative	Negative	NA
15	Positive	Negative	Negative	Negative	NA
16	Positive	Negative	Negative	Negative	NA
17	Positive	Negative	Negative	Negative	NA
18	Positive	Negative	Negative	Negative	NA
19	Positive	Negative	Negative	Negative	NA
20	Positive	Negative	Negative	Negative	NA
21	Positive	Negative	Negative	Negative	NA
22	Positive	Negative	Negative	Negative	NA
23	Positive	Negative	Negative	Negative	NA
26	Positive	Negative	Negative	Negative	NA
27	Positive	Negative	Negative	Negative	NA
28	Positive	Negative	Negative	Negative	NA
29	Positive	Negative	Negative	Negative	NA
30	Positive	Negative	Negative	Negative	NA
31	Positive	Negative	Negative	Negative	NA
36	Positive	Negative	Negative	Negative	NA
37	Positive	Negative	Negative	Negative	NA
38	Positive	Negative	Negative	Negative	NA
40	Positive	Negative	Negative	Negative	NA

41	Positive	Negative	Negative	Negative	NA
42	Positive	Negative	Negative	Negative	NA
43	Positive	Negative	Negative	Negative	NA
44	Positive	Negative	Negative	Negative	NA
45	Positive	Positive	NA	Positive	HPV16
47	Positive	Negative	Negative	Negative	NA
48	Positive	Negative	Negative	Negative	NA
51	Positive	Negative	Negative	Negative	NA
53	Positive	Negative	Negative	Negative	NA
55	Positive	Positive	NA	Positive	HPV 33
58	Positive	Negative	Negative	Negative	NA
59	Positive	Negative	Negative	Negative	NA
60	Positive	Negative	Negative	Negative	NA
61	Positive	Negative	Negative	Negative	NA
62	Positive	Negative	Negative	Negative	NA
63	Positive	Negative	Negative	Negative	NA
65	Positive	Negative	Negative	Negative	NA
66	Positive	Negative	Negative	Negative	NA
67	Positive	Negative	Negative	Negative	NA
68	Positive	Negative	Negative	Negative	NA
69	Positive	Negative	Negative	Negative	NA
70	Positive	Negative	Negative	Negative	NA
71	Positive	Negative	Negative	Negative	NA
73	Positive	Negative	Negative	Negative	NA
74	Weak Positive	Negative	Negative	Negative	NA
75	Positive	Negative	Negative	Negative	NA
76	Positive	Negative	Negative	Negative	NA
77	Positive	Negative	Negative	Negative	NA
79	Positive	Negative	Negative	Negative	NA
80	Positive	Negative	Negative	Negative	NA
81	Positive	Negative	Negative	Negative	NA
82	Positive	Negative	Negative	Negative	NA
83	Positive	Negative	Negative	Negative	NA
84	Positive	Negative	Weak positive	Weak Positive	HPV16
85	Positive	Negative	Negative	Negative	NA
86	Positive	Negative	Negative	Negative	NA
87	Positive	Negative	Negative	Negative	NA
88	Positive	Positive	NA	Positive	HPV16
89	Positive	Negative	Negative	Negative	NA
90	Positive	Negative	Negative	Negative	NA

91	Positive	Negative	Negative	Negative	NA
92	Positive	Negative	Negative	Negative	NA
94	Positive	Negative	Negative	Negative	NA
95	Positive	Negative	Negative	Negative	NA
97	Positive	Negative	Negative	Negative	NA
98	Positive	Negative	Negative	Negative	NA
99	Positive	Negative	Negative	Negative	NA
101	Weak Positive	Negative	Negative	Negative	NA
102	Positive	Negative	Negative	Negative	NA
104	Positive	Negative	Negative	Negative	NA
108	Positive	Negative	Negative	Negative	NA
109	Positive	Negative	Negative	Negative	NA
110	Positive	Negative	Negative	Negative	NA
112	Positive	Negative	Negative	Negative	NA
113	Positive	Negative	Negative	Negative	NA
114	Positive	Negative	Negative	Negative	NA
115	Positive	Negative	Negative	Negative	NA
116	Positive	Negative	Negative	Negative	NA
117	Positive	Negative	Negative	Negative	NA
118	Positive	Negative	Negative	Negative	NA
121	Positive	Negative	Negative	Negative	NA
123	Positive	Negative	Negative	Negative	NA
127	Positive	Negative	Negative	Negative	NA
128	Positive	Negative	Negative	Negative	NA
129	Positive	Negative	Negative	Negative	NA
131	Positive	Negative	Negative	Negative	NA
132	Positive	Negative	Negative	Negative	NA
133	Positive	Negative	Weak positive	Weak Positive	HPV16
134	Positive	Positive	NA	Positive	HPV16
135	Positive	Weak Positive	NA	Weak Positive	HPV16
136	Positive	Positive	NA	Positive	HPV16
137	Positive	Positive	NA	Positive	HPV16
139	Weak Positive	Negative	Negative	Negative	NA
140	Positive	Positive	NA	Positive	HPV16
142	Positive	Positive	NA	Positive	HPV16
143	Positive	Positive	NA	Positive	HPV16
144	Positive	Positive	NA	Positive	HPV16
145	Positive	Positive	NA	Positive	HPV16
146	Weak Positive	Negative	Positive	Positive	HPV16

147	Weak Positive	Weak Positive	Positive	Positive	HPV16
148	Positive	Positive	NA	Positive	HPV16
149	Positive	Weak Positive	Positive	Positive	HPV16
150	Positive	Weak Positive	Positive	Positive	HPV16
153	Positive	Positive	Positive	Positive	HPV16
154	Positive	Positive	NA	Positive	HPV16
155	Positive	Negative	Negative	Negative	NA
156	Positive	Positive	NA	Positive	HPV16
157	Positive	Negative	Negative	Negative	NA
158	Positive	Negative	Positive	Positive	HPV 16
159	Positive	Positive	NA	Positive	HPV16
161	Positive	Negative	Negative	Negative	NA
162	Positive	Negative	Negative	Negative	NA
163	Positive	Negative	Negative	Negative	NA
164	Positive	Negative	Negative	Negative	NA
165	Positive	Negative	Negative	Negative	NA
166	Positive	Negative	Negative	Negative	NA
167	Positive	Negative	Negative	Negative	NA
168	Positive	Positive	NA	Positive	HPV16
169	Positive	Positive	NA	Positive	HPV16
170	Positive	Positive	NA	Positive	HPV16
171	Positive	Negative	Negative	Negative	NA
172	Positive	Negative	Negative	Negative	NA
173	Positive	Negative	Negative	Negative	NA
174	Positive	Positive	NA	Positive	HPV16
175	Positive	Negative	Negative	Negative	NA
176	Weak Positive	Negative	Positive	Positive	HPV16
177	Positive	Negative	Negative	Negative	NA
178	Positive	Negative	Negative	Negative	NA
179	Positive	Positive	NA	Positive	HPV16
180	Positive	Weak Positive	Positive	Positive	HPV16
181	Positive	Positive	NA	Positive	HPV16
183	Positive	Positive	Positive	Positive	HPV16
184	Positive	Negative	Negative	Negative	NA
185	Positive	Negative	Negative	Negative	NA
186	Positive	Positive	NA	Positive	HPV16
187	Positive	Negative	Negative	Negative	NA
188	Weak Positive	Weak Positive	Positive	Positive	HPV16
189	Positive	Negative	Positive	Positive	HPV16

190	Positive	Positive	Positive	Positive	HPV16
191	Positive	Negative	Negative	Negative	NA
192	Positive	Positive	NA	Positive	HPV16
193	Positive	Weak Positive	Positive	Positive	HPV16
194	Positive	Negative	Negative	Negative	NA
195	Positive	Weak Positive	Positive	Positive	HPV16
196	Positive	Positive	NA	Positive	HPV16
197	Positive	Negative	Negative	Negative	NA
198	Positive	Positive	NA	Positive	HPV16
199	Positive	Negative	Negative	Negative	NA
201	Positive	Negative	Negative	Negative	NA
204	Positive	Negative	Negative	Negative	NA
205	Positive	Negative	Negative	Negative	NA
206	Weak Positive	Negative	Negative	Negative	NA
207	Weak Positive	Negative	Negative	Negative	NA
208	Weak Positive	Negative	Negative	Negative	NA
209	Positive	Negative	Negative	Negative	NA
210	Positive	Negative	Negative	Negative	NA
211	Positive	Negative	Negative	Negative	NA
212	Weak Positive	Negative	Negative	Negative	NA
213	Positive	Negative	Negative	Negative	NA
214	Positive	Negative	Negative	Negative	NA
215	Positive	Negative	Negative	Negative	NA
216	Positive	Negative	Negative	Negative	NA
218	Positive	Negative	Negative	Negative	NA
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224	Positive	Negative	Negative	Negative	NA
225	Weak Positive	Negative	Negative	Negative	NA
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234	Positive	Negative	Negative	Negative	NA
237	Positive	Negative	Negative	Negative	NA

238	Positive	Negative	Negative	Negative	NA
239	Positive	Negative	Negative	Negative	NA
240	Positive	Negative	Negative	Negative	NA
241	Positive	Negative	Negative	Negative	NA
242	Positive	Negative	Negative	Negative	NA
243	Positive	Negative	Negative	Negative	NA
244	Positive	Negative	Negative	Negative	NA
245	Positive	Negative	Negative	Negative	NA
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256	Positive	Negative	Negative	Negative	NA
257	Positive	Negative	Negative	Negative	NA
258	Positive	Negative	Negative	Negative	NA
259	Positive	Negative	Negative	Negative	NA
260	Positive	Negative	Negative	Negative	NA
261	Positive	Negative	Negative	Negative	NA
262	Positive	Negative	Negative	Negative	NA
263	Positive	Negative	Negative	Negative	NA
266	Positive	Negative	Negative	Negative	NA
267	Positive	Negative	Negative	Negative	NA
268	Positive	Negative	Negative	Negative	NA
269	Positive	Negative	Negative	Negative	NA
270	Positive	Negative	Negative	Negative	NA
271	Positive	Negative	Negative	Negative	NA
273	Positive	Negative	Weak positive	Weak Positive	HPV16
274	Positive	Negative	Negative	Negative	NA
275	Positive	Negative	Negative	Negative	NA
276	Positive	Negative	Negative	Negative	NA
277	Positive	Negative	Negative	Negative	NA
278	Positive	Negative	Negative	Negative	NA
279	Positive	Negative	Negative	Negative	NA
281	Positive	Negative	Negative	Negative	NA
282	Positive	Negative	Negative	Negative	NA
284	Positive	Negative	Negative	Negative	NA
285	Weak Positive	Negative	Negative	Negative	NA
286	Positive	Negative	Negative	Negative	NA
287	Positive	Negative	Negative	Negative	NA

288	Positive	Negative	Negative	Negative	NA
289	Weak Positive	Negative	Negative	Negative	NA
293	Weak Positive	Negative	Negative	Negative	NA
294	Positive	Negative	Negative	Negative	NA
295	Positive	Negative	Negative	Negative	NA
297	Positive	Negative	Negative	Negative	NA
298	Positive	Positive	NA	Positive	HPV16
299	Positive	Negative	Negative	Negative	NA
300	Positive	Negative	Negative	Negative	NA
301	Positive	Negative	Negative	Negative	NA
302	Positive	Positive	NA	Positive	HPV16
303	Positive	Negative	Negative	Negative	NA
305	Positive	Negative	Negative	Negative	NA
306	Positive	Negative	Negative	Negative	NA
307	Positive	Negative	Negative	Negative	NA
308	Weak Positive	Negative	Negative	Negative	NA
311	Positive	Negative	Negative	Negative	NA
312	Positive	Negative	Negative	Negative	NA
314	Positive	Negative	Negative	Negative	NA
316	Positive	Negative	Negative	Negative	NA
319	Positive	Positive	NA	Positive	HPV16
320	Positive	Negative	Negative	Negative	NA
322	Positive	Negative	Negative	Negative	NA
324	Weak Positive	Positive	NA	Positive	HPV16
326	Positive	Positive	NA	Positive	HPV 18
328	Positive	Negative	Positive	Positive	HPV16
329	Positive	Negative	Negative	Negative	NA
331	Weak Positive	Negative	Negative	Negative	NA
332	Positive	Negative	Positive	Positive	HPV16
334	Weak Positive	Negative	Negative	Negative	NA
335	Positive	Negative	Positive	Positive	HPV16
337	Positive	Negative	Negative	Negative	NA
339	Positive	Negative	Negative	Negative	NA
340	Positive	Negative	Negative	Negative	NA
342	Positive	Negative	Negative	Negative	NA
343	Positive	Negative	Negative	Negative	NA
344	Positive	Negative	Negative	Negative	NA
346	Positive	Negative	Negative	Negative	NA
347	Positive	Negative	Negative	Negative	NA

348	Positive	Positive	Negative	Positive	HPV16
349	Positive	Negative	Negative	Negative	NA
350	Positive	Negative	Negative	Negative	NA
351	Weak Positive	Negative	Negative	Negative	NA
352	Positive	Negative	Negative	Negative	NA
353	Positive	Negative	Negative	Negative	NA
354	Positive	Negative	Negative	Negative	NA
355	Positive	Negative	Negative	Negative	NA
356	Positive	Negative	Negative	Negative	NA
357	Positive	Negative	Negative	Negative	NA
358	Positive	Negative	Negative	Negative	NA
359	Positive	Negative	Negative	Negative	NA
361	Positive	Negative	Negative	Negative	NA
362	Positive	Negative	Negative	Negative	NA
364	Positive	Negative	Negative	Negative	NA
365	Positive	Negative	Negative	Negative	NA
366	Positive	Negative	Negative	Negative	NA
367	Positive	Negative	Negative	Negative	NA
368	Positive	Negative	Negative	Negative	NA
369	Positive	Negative	Negative	Negative	NA
370	Positive	Negative	Negative	Negative	NA
371	Positive	Negative	Negative	Negative	NA
372	Positive	Negative	Negative	Negative	NA
373	Positive	Negative	Negative	Negative	NA
374	Positive	Negative	Negative	Negative	NA
376	Positive	Negative	Negative	Negative	NA

a. ‘NA’ – Not Applicable. Only samples that were negative for HPV L1 were required to be put through PCR for HPV E1 and only samples positive for either HPV L1 or E1 were put through PCR for specific HPV types.

APPENDIX C

Scoring of IHC results for EBP50 expression

Table A: Scoring of IHC results for EbP50 expression

Specimen ID	EBP50 membrane staining	EBP50 cytoplasm staining	Membrane/Cytoplasmic EBP50 staining	EBP50 expression pattern
99	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
163	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	1
329	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
89	Mixed staining - traces to positive	Moderate staining	Membrane mixed/Cytoplasm+ve	3
88	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
87	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
85	Mixed staining - traces to positive	Moderate staining	Membrane mixed/Cytoplasm+ve	3
86	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
83	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
81	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
129	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
128	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
158	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
184	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
113	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
328	Traces of membrane staining	Negligible or weak staining	Membrane+ve/Cytoplasm-ve	4
256	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
135	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
162	Membrane excluded	Mixed staining - Moderate to weak/negligible	Membrane-ve/Cytoplasm mixed	3
155	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
140	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
143	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
357	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
250	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
159	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
148	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
348	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
147	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
136	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
168	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
169	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
142	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
171	Traces of membrane staining	Negligible or weak staining	Membrane+ve/Cytoplasm-ve	4
170	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
212	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
181	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
347	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
320	Membrane excluded	Mixed staining - Moderate to weak/negligible	Membrane-ve/Cytoplasm mixed	3
157	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1

233	Membrane excluded	Strong cytoplasmic staining	Membrane-ve/Strong cytoplasmic staining	4
153	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
183	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
134	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
156	Membrane excluded	Mixed staining - Moderate to weak/negligible	Membrane-ve/Cytoplasm mixed	3
204	Mixed staining - traces to negative	Moderate staining	Membrane mixed/Cytoplasm+ve	3
133	Membrane excluded	Mixed staining - Moderate to weak/negligible	Membrane-ve/Cytoplasm mixed	3
206	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
194	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
161	Traces of membrane staining	Moderate staining	Membrane+ve/Cytoplasm+ve	4
139	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
193	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
188	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
192	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
175	Mixed staining - Positive to excluded	Moderate cytoplasmic staining	Membrane mixed/Cytoplasm+ve	3
178	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
144	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
154	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
149	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
166	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
180	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
195	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
176	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
174	Membrane excluded	Mixed staining - Strong to weak/negligible	Membrane-ve/Cytoplasm mixed	3
191	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
145	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
179	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
189	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
172	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
146	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
198	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
165	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
324	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
187	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
14	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
15	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
186	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
6	(Mixed staining - Positive to excluded	Moderate cytoplasmic staining	Membrane mixed/Cytoplasm+ve	3
196	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
199	Mixed staining - Positive to excluded	C+/-(Mixed staining - Moderate to weak/negligible	Membrane mixed/Cytoplasm mixed	3
173	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
58	Membrane excluded	Moderate cytoplasmic	Membrane-ve/Cytoplasm+ve	1

		staining		
45	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
44	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
43	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
167	Membrane excluded	Moderate staining	Membrane-ve/Cytoplasm+ve	1
185	Membrane excluded	Mixed staining - Moderate to weak/negligible	Membrane-ve/Cytoplasm mixed	3
314	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
312	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
372	Inconclusive	Moderate cytoplasmic staining	Membrane mixed/Cytoplasm+ve	3
307	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
306	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
305	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
302	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
303	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
301	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
299	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
298	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
311	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
197	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
288	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
337	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
278	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
322	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
211	Mixed staining- traces to negative	Moderate cytoplasmic staining	Membrane mixed/Cytoplasm+ve	3
221	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
219	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
361	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
213	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
274	Membrane excluded	Strong cytoplasmic staining	Membrane-ve/Strong cytoplasmic staining	4
164	Membrane excluded	Mixed staining - Moderate to faint/negligible	Membrane-ve/Cytoplasm mixed	3
359	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
260	Membrane excluded	Strong cytoplasmic staining	Membrane-ve/Strong cytoplasmic staining	4
365	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
350	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
225	Traces of membrane staining	Mixed staining - Moderate to faint/negligible	Membrane+ve/Cytoplasm mixed	3
276	Mixed staining - traces	Strong cytoplasmic staining	Membrane mixed/Strong	3

	to positive		cytoplasmic staining	
373	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
367	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
177	Membrane excluded	Mixed staining - Moderate to faint/negligible	Membrane-ve/Cytoplasm mixed	3
370	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
351	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
326	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
371	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
376	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
369	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
137	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
16	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
17	Membrane excluded	Mixed staining - Moderate to faint/negligible	Membrane-ve/Cytoplasm mixed	3
5	Mixed staining - Positive to excluded	Moderate cytoplasmic staining	Membrane mixed/Cytoplasm+ve	3
4	Traces of membrane staining	Moderate cytoplasmic staining	Membrane+ve/Cytoplasm+ve	4
2	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
1	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
190	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
55	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
53	Membrane excluded	Mixed staining - Strong to moderate	Membrane-ve/Cytoplasm mixed	3
101	Mixed staining - Positive to excluded	Moderate cytoplasmic staining	Membrane mixed/Cytoplasm+ve	3
98	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
84	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
201	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
308	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
239	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
285	Membrane excluded	Strong cytoplasmic staining	Membrane-ve/Strong cytoplasmic staining	4
284	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
279	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
335	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
364	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
334	Membrane excluded	Mixed staining - Moderate to faint/negligible	Membrane-ve/Cytoplasm mixed	3
266	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
263	Membrane excluded	Strong cytoplasmic staining)	Membrane-ve/Strong cytoplasmic staining	4
220	Membrane excluded	Mixed staining-Strong to negligible/weak	Membrane-ve/Cytoplasm mixed	3
271	Mixed staining - Positive to excluded	Strong cytoplasmic staining	Membrane mixed/Strong cytoplasmic staining	3

273	Mixed staining - Positive to excluded	Strong cytoplasmic staining	Membrane mixed/Strong cytoplasmic staining	3
281	Traces of membrane staining	Negligible or weak staining	Membrane+ve/Cytoplasm-ve	4
332	Membrane excluded	Negligible or weak staining	Membrane-ve/Cytoplasm-ve	2
319	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1
331	Membrane excluded	Moderate cytoplasmic staining	Membrane-ve/Cytoplasm+ve	1

1- Predominantly cytoplasmic EBP50 expression

2- Weak/negligible cytoplasmic EBP50 expression

3- Mixed-heterogeneous EBP50 expression

4- Other less common patterns of EBP50 expression

APPENDIX D

Microscopic images of EBP50 immunohistochemistry

The enclosed CD contains images of EBP50 expression in the cohort specimens. The images were taken at x 10 and x 40 magnification and are organized into folders labeled with the specimen ID. The images may be viewed in tandem with the information in Appendix C using the specimen ID.

APPENDIX E

Oral presentations

National

I have presented annually at the University of Dundee Postgraduate Research Symposium.

International

Current practice trends in HPV testing for head and neck squamous cell carcinomas: A Scotland-wide online survey. Presented at the WHO International Agency for Research on Cancer meeting on Emerging issues in Head and Neck Cancer in Manduria, Italy, June 2014.